

**Formation of Biogenic Iron-Oxides (BIOS) and Their Susceptibility to Microbial
Iron Reduction and Fermentation Under Anoxic Conditions**

Dissertation

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Summary

Biogenic Iron (Oxyhydr)oxides (BIOS) are organo-minerals formed as a result of Fe(II) oxidation by Fe(II)-Oxidizing Bacteria (FeOB). The mineral part is typically poorly crystalline Fe(III) (oxyhydr)oxides (*e.g.*, ferrihydrite) and goethite, while the organic part is mainly polysaccharides moieties and cells. Their composition and precipitation vary depending on the metabolism of the FeOB. Nitrate-dependent FeOB show high degree of cell encrustation, whereas phototrophic FeOB have developed mechanisms to avoid encrustation because encrustation prevents the photon uptake for photosynthesis. BIOS play an important role in the environment as the Fe(III) can be used as electron acceptor while the associated carbon can act as an electron donor and/or carbon source. Moreover, BIOS are potential biosignatures (*e.g.*, microfossils or molecular). Here we present a general overview of FeOB and FeRB metabolisms, BIOS and their reactivity, with a focus on their use as biosignatures of past and present life on Earth, and in other Fe-rich bodies, such as Mars and icy moons (**Chapter 2**).

However, we acknowledge that little is known on the bioavailability of the Fe and C for Fe(III)-reducing bacteria, the effect of the BIOS source and the transformation products. Therefore, in this PhD thesis we chose three FeOB representatives of (a) phototrophic metabolism (*Rhodopseudomonas palustris* TIE-1), (b) chemoautotrophy (enrichment culture KS) and (c) mixotrophy (*Acidovorax* sp. BoFeN1). We described the mineralogy and organic bonds of their BIOS and test their bioavailability for Fe(III) reduction and C oxidation using two Fe(III)-reducing bacteria (FeRB) and fermentative microbes. We used *Geobacter sulfurreducens* and *Shewanella oneidensis* MR-1, which are representatives of two different Fe(III) reduction mechanisms, being direct cell-mineral contact and via electron shuttles, respectively. To test the C degradation we enriched fermentative microbes from sediments of Lake Constance using different carbon sources (yeast extract, autoclaved cells of *S. oneidensis* and formaldehyde fixed cells of *S. oneidensis*).

We found lepidocrocite and poorly crystalline Fe oxides to be the dominant minerals and carbohydrates and Fe-C bonds to be present in similar proportion in the three BIOS, although the total organic carbon was 2.21 wt%, 0.2 wt % and 1.29 wt % for TIE-1, KS and BoFeN1 respectively. Reduction experiments showed that the presence of available organic carbon was the main factor affecting the extent of reduction, while the reduction rate highly depended on the bacteria involved (both FeOB and FeRB). In the presence of an additional carbon source (acetate/lactate for *G. sulfurreducens* and *S. oneidensis* MR-1, respectively) the BIOS-Fe(III) were reduced to a 100%,

although 3-10 times faster by *G. sulfurreducens*. Without additional C, the maximum extent of reduction was 50% and *G. sulfurreducens* reduced the Fe(III) 2-7 faster (**Chapter 3**).

The BIOS-Fe(III) reduction resulted in mineral transformation to vivianite in all cases. Interestingly, the reduction of the BIOS from BoFeN1, the FeOB with the highest degree of encrustation, resulted in cells being embedded in the newly formed vivianite (as seen by cryo-SEM). Then, we used high resolution techniques (e.g., cryo-SIM-SXT) and confirmed that the Fe(III) (oxyhydr)oxides in the cell wall act as nucleation sites for the formation of vivianite, which thus grows around the cells and preserves it (**Chapter 4**).

Finally, because without additional organic carbon the FeRB did not reach a 100% extent of reduction, suggesting that the BIOS-C might be complex for complete FeRB degradation, we enriched fermenting microbes from sediments of Lake Constance to assess the biodegradability of the BIOSO-associated C for specialized C degraders. However, we cannot confidently confirm degradation of the BIOS-associated C, and more tests will be required to assess this knowledge gap (**Chapter 5**).

Overall, this work broadened our knowledge of the bioavailability of the mineral component of BIOS as electron acceptor for FeRB in different soils and of the organic component for fermentation. Moreover, it strengthened the potential of Fe minerals for preservation of organic matter in the rock record, despite early diagenetic events.

Zusammenfassung

Biogene Eisen-(Oxyhydr)oxide (BIOS) sind Organo-Mineralen, die infolge der Oxidation von Fe(II) durch Fe(II)-oxidierende Bakterien (FeOB) gebildet werden. Der mineralische Anteil besteht typischerweise aus schlecht kristallinen Fe(III)-(Oxyhydr)oxiden (z. B. Ferrihydrit) und Goethit, während der organische Anteil hauptsächlich aus Polysaccharid-Einheiten und Zellen besteht. Zusammensetzung und Ausfällung der BIOS variieren in Abhängigkeit vom Stoffwechsel der FeOB. Nitratabhängige FeOB zeigen einen hohen Grad an Zellverkrustung, wohingegen phototrophe FeOB Mechanismen entwickelt haben, um eine Verkrustung zu vermeiden, da diese die Aufnahme von Photonen für die Photosynthese verhindert. BIOS spielen eine wichtige Rolle in der Umwelt, da Fe(III) als Elektronenakzeptor genutzt werden kann, während der assoziierte Kohlenstoff als Elektronendonator und/oder Kohlenstoffquelle dient. Darüber hinaus stellen BIOS potenzielle Biosignaturen dar (z. B. Mikrofossilien oder molekulare Signaturen). In dieser Arbeit geben wir einen allgemeinen Überblick über die Stoffwechselwege von FeOB und Fe(III)-reduzierenden Bakterien (FeRB), über BIOS und deren Reaktivität, mit besonderem Fokus auf ihre Nutzung als Biosignaturen vergangener und heutiger Lebensformen auf der Erde sowie auf anderen eisenreichen Himmelskörpern, wie dem Mars und eisbedeckten Monden (**Kapitel 2**).

Allerdings ist bislang wenig über die Bioverfügbarkeit von Eisen und Kohlenstoff für Fe(III)-reduzierende Bakterien, den Einfluss der BIOS-Quelle sowie über die entstehenden Umwandlungsprodukte bekannt. Daher wurden in dieser Dissertation drei FeOB-Vertreter ausgewählt: (a) phototropher Stoffwechsel (*Rhodopseudomonas palustris* TIE-1), (b) chemoautotropher Stoffwechsel (Anreicherungskultur KS) und (c) mixotropher Stoffwechsel (*Acidovorax* sp. BoFeN1). Die Mineralogie und organischen Bindungen ihrer BIOS wurden charakterisiert und deren Bioverfügbarkeit für die Fe(III)-Reduktion und C-Oxidation mithilfe von zwei Fe(III)-reduzierenden Bakterien sowie fermentativen Mikroorganismen untersucht. Hierzu wurden *Geobacter sulfurreducens* und *Shewanella oneidensis* MR-1 eingesetzt, die zwei unterschiedliche Mechanismen der Fe(III)-Reduktion repräsentieren, nämlich direkten Zell-Mineral-Kontakt bzw. Elektronentransfer über Elektroschuttles. Zur Untersuchung des Kohlenstoffabbaus wurden fermentative Mikroorganismen aus Sedimenten des Bodensees angereichert, wobei verschiedene Kohlenstoffquellen verwendet wurden (Hefeextrakt, autoklavierte Zellen von *S. oneidensis* sowie formaldehydfixierte Zellen von *S. oneidensis*).

Als dominante Minerale wurden Lepidokrokit und schlecht kristalline Eisenoxide identifiziert. Kohlenhydrate und Fe-C-Bindungen waren in allen drei BIOS in ähnlichen Anteilen vorhanden,

obwohl der Gesamtgehalt an organischem Kohlenstoff 2,21 Gew.-%, 0,2 Gew.-% bzw. 1,29 Gew.-% für TIE-1, KS und BoFeN1 betrug. Reduktionsexperimente zeigten, dass das Vorhandensein von verfügbarem organischem Kohlenstoff der wichtigste Faktor für das Ausmaß der Reduktion war, während die Reduktionsrate stark von den beteiligten Bakterien (sowohl FeOB als auch FeRB) abhing. In Anwesenheit einer zusätzlichen Kohlenstoffquelle (Acetat bzw. Laktat für *G. sulfurreducens* bzw. *S. oneidensis* MR-1) wurden die BIOS-Fe(III) vollständig (100 %) reduziert, wobei die Reduktion durch *G. sulfurreducens* 3- bis 10-mal schneller erfolgte. Ohne zusätzliche Kohlenstoffquelle betrug das maximale Reduktionsausmaß 50 %, wobei *G. sulfurreducens* die Fe(III)-Reduktion 2- bis 7-mal schneller durchführte (**Kapitel 3**).

Die Reduktion von BIOS-Fe(III) führte in allen Fällen zu einer Mineralumwandlung zu Vivianit. Interessanterweise führte die Reduktion der BIOS von BoFeN1, dem FeOB mit dem höchsten Grad an Zellverkrustung, dazu, dass die Zellen in den neu gebildeten Vivianit eingebettet wurden, wie mittels Kryo-SEM gezeigt werden konnte. Mithilfe hochauflösender Techniken (z. B. Kryo-SIM-SXT) wurde anschließend bestätigt, dass die Fe(III)-(Oxyhydr)oxide in der Zellwand als Nukleationsstellen für die Bildung von Vivianit fungieren, welcher somit um die Zellen herum wächst und diese konserviert (**Kapitel 4**).

Schließlich wurden vor dem Hintergrund, dass ohne zusätzliche organische Kohlenstoffquelle kein vollständiges Reduktionsausmaß von 100 % durch die FeRB erreicht wurde – was darauf hindeutet, dass der BIOS-gebundene Kohlenstoff für einen vollständigen Abbau durch FeRB zu komplex sein könnte – fermentative Mikroorganismen aus Sedimenten des Bodensees angereichert, um die biologische Abbaubarkeit des BIOS-assoziierten Kohlenstoffs durch spezialisierte Kohlenstoffabbauer zu untersuchen. Eine eindeutige Bestätigung des Abbaus des BIOS-assoziierten Kohlenstoffs konnte jedoch nicht erzielt werden, sodass weitere Untersuchungen erforderlich sind, um diese Wissenslücke zu schließen (**Kapitel 5**).

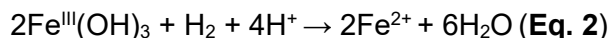
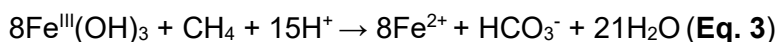
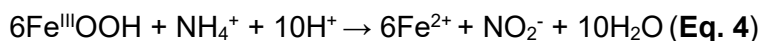
Insgesamt erweiterte diese Arbeit unser Verständnis der Bioverfügbarkeit der mineralischen Komponente von BIOS als Elektronenakzeptor für FeRB in unterschiedlichen Böden sowie der organischen Komponente für fermentative Prozesse. Darüber hinaus unterstreicht sie das Potenzial von Eisenmineralen zur Konservierung organischer Substanz im Gesteinsarchiv, trotz früher diagenetischer Prozesse.

Chapter 1. Introduction

Iron biogeochemistry

Iron (Fe) is a highly abundant redox-active element and its redox cycle is closely linked to the transformation of carbon, nitrogen, sulfur, and trace metals in the environment (Kappler et al., 2021). Fe has a range of oxidation states from -2 to +6. However it predominantly occurs in two oxidation states: ferric [Fe(III)] and ferrous [Fe(II)] iron. Fe(III) commonly forms in oxic environments with pH above 5 as poorly soluble Fe(III) (oxyhydr)oxide minerals. Fe(II) occurs in suboxic or anoxic settings such as stratified lakes, groundwater systems, and sediment porewaters (Bhattacharyya et al., 2018; Huang et al., 2021). Abiotic reduction of Fe(III) can occur by reaction with sulfur species, organic compounds, hydrogen, Fe(II) (autocatalytic) or photochemically (Barbeau, 2006; Poulton et al., 2004; Williams & Scherer, 2004). At the same time, abiotic oxidation of Fe(II) is possible by reaction with reactive oxygen species (e.g., H₂O₂, O₂), nitrogen species (nitrate and nitrite), manganese (Mn(IV) oxides) or photochemically (Kappler et al., 2021; Stumm & Lee, 1961). Furthermore, the transformation between Fe(II) and Fe(III) occurs biotically, facilitated by Fe(III)-reducing bacteria (FeRB) and Fe(II)-oxidizing bacteria (FeOB) (Blöthe & Roden, 2009; Laufer et al., 2016).

FeRB are found in marine and freshwater sediments, where they reduce Fe(III) using organic carbon (OC), methane, hydrogen or ammonium as electrons donors (**Eq. 1, 2, 3, 4**) (Glodowska et al., 2020; Kappler et al., 2021; Melton et al., 2014). They can reduce different Fe(III) minerals (e.g., ferrihydrite and lepidocrocite) with varying rate and extent of reduction depending on the properties of the minerals, such as phase, crystallinity, size and specific surface area. As a result, the Fe(III) minerals are transformed into (mixed valent) Fe(II) minerals including siderite, vivianite and magnetite.

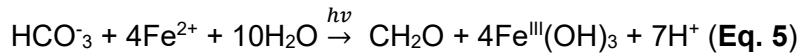
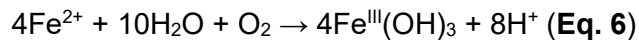
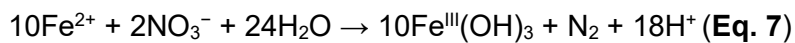
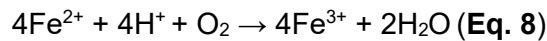


The low solubility of Fe(III) (10⁻¹⁰ M) means that most organisms rely on siderophores to extract solid-phase Fe for its assimilation into biomass (Ahmed & Holmström, 2014). In contrast, FeRB use membrane proteins (c-type cytochrome) in the cell surface to reduce Fe(III) via direct cell-mineral contact (Shi et al., 2009). Some FeRB possess pili or nanowires that transport the

electrons to the electron acceptor which allows the reduction to occur at larger distances (few μm) (Reguera et al., 2006; Smith et al., 2013). Another strategy is the use of electron shuttles, which themselves are reduced enzymatically, allowing for electron transfer to the final acceptor abiotically (Bai et al., 2020). This strategy increases the distance of reduction to a cm-scale (Bai et al., 2020).

Geobacter and *Shewanella* are two model genera of FeRB used to study the biogenic reduction of Fe(III). They have a fundamental difference in their primary reduction mechanism, *Geobacter* typically uses direct cell-mineral contact, whereas *Shewanella* produces electron shuttles. Therefore, we chose two representative members of each genus to study the reduction of the Fe(III) associated to biogenic iron(III) oxides (BIOS): *S. oneidensis* MR-1 (Myers & Nealson, 1988) and *G. sulfurreducens* (Caccavo et al., 1994; Tabares et al., 2020). *S. oneidensis* utilizes self-made flavins or oxidized organic matter from the environment as electron shuttles (Marsili et al., 2008). *G. sulfurreducens* possess multiple *c*-type cytochromes, mainly in the outer membrane, and a type IV pili through which the electron transfer is carried out (Shi et al., 2007).

Fe(II) is soluble and therefore a more bioavailable form of Fe for assimilatory metabolisms. It is also used in dissimilatory metabolisms, oxidized biotically by FeOB. Multiple Fe(II) oxidation metabolisms are known. Photoferrotrophs utilize sunlight energy and electrons from Fe(II) to fix CO_2 (**Eq. 5**) and are found in sediments and water bodies (Camacho et al., 2017; Widdel et al., 1993). Microaerophiles outcompete abiotic Fe(II) oxidation in environments (**Eq. 6**) where the concentration of O_2 is between 3 and 50 μM O_2 (Druschel et al., 2008; Lueder et al., 2022; Maisch et al., 2019). Nitrate-reducing Fe(II)-oxidizing bacteria (NRFeOB) obtain electrons from Fe(II) couple to the reduction of nitrate (NO_3^-) to gain energy (**Eq. 7**) and inhabit modern freshwater sediments, groundwater and soils (Liu et al., 2019; Straub et al., 1996). Acidophilic FeOB thrive under acidic conditions ($\text{pH} < 4$) and are obligate aerobes, using oxygen as electron acceptor (**Eq. 8**) (Johnson & Hallberg, 2008; Bird et al., 2011; Johnson, 1998).



Fe and C associations

In the environment, Fe(III) oxides act as carbon sink. They absorb organic carbon in the surface leading to complex structures that stabilize the organic carbon in soils and sediments for long periods of time (Giannetta et al., 2019; Guo et al., 2025; Jia et al., 2024). These structures alter the reactivity and mobility of carbon. However, they are susceptible to transformation by microorganisms. On one hand, FeRB can use the Fe(III) as electron acceptor with the subsequent minerals transformation or Fe(III) release (Kappler et al., 2021). On the other hand, the released carbon can be used as carbon source for fermentative microorganisms with the subsequent transformation and release of CO₂ and CH₄ (Patzner et al., 2022; Voggenreiter et al., 2025). To complete the microbial Fe-C cycle, autotrophic FeOB can fix the CO₂. The oxidation of Fe(II) by FeOB produces Fe(III) minerals known as biogenic iron(III) oxides (BIOS), which are another form of Fe-C association (Emerson et al., 2010; Hedrich et al., 2011; Toner et al., 2009). The formation and degradation of BIOS play an important role in the cycle of both Fe and C (Chen et al., 2024, 2024).

BIOS

BIOS are composed of a mixture of Fe minerals, organic matter and cells. In general, the minerals are ferrihydrite, goethite and amorphous Fe(III) phosphates (Ferris, 2005; Langley et al., 2009; Miot et al., 2009). The organic part are biopolymers, mainly lipids and polysaccharide moieties (Chan et al., 2009; Sowers et al., 2019; Whitaker et al., 2021). The morphology of these BIOS is highly dependent on the FeOB and their corresponding ecological niches (**Fig. 1**).

Some NRFeOB become encrusted by the formed Fe(III) minerals that precipitate in the cell membrane due to attraction of positive charged Fe ions to the negatively charged membrane (Miot et al., 2009). Although this encrustation could be beneficial, by preventing cell dehydration, it prevents the uptake of nutrients and leads to cell death (Knoll et al., 2012). Microaerophilic FeOB produce BIOS typically composed of poorly crystalline ferrihydrite, lepidocrocite, goethite or akageneite (Hallbeck & Pedersen, 1990; Krepski et al., 2013; Laufer et al., 2017). Particularly, some species (*e.g.*, *Gallionella ferruginea*, *Mariprofundus ferrooxydans*) produce extracellular polymeric substances (EPS) where the Fe(III) minerals precipitate and the EPS serves as a template for the formation of morphologies such as twisted stalks, tubular sheaths, granular or dreadlock-like structures (Chan et al., 2011, 2016; Hallberg, 2004). Photoferrotrophs also produce negatively charged EPS that complex the Fe(III) minerals to prevent the precipitation on the cell surface (Chan et al., 2011; Wu et al., 2014). In this case the minerals are mainly poorly crystalline

minerals and goethite (Jiao et al., 2005). Moreover, some species of photoferrotrophs modify the composition of the cell surface to cause an electrostatic repulsion between the cell and the minerals (Stukalov et al., 2008; Thompson et al., 2019) or acidify the surrounding microenvironment to keep the Fe(III) dissolved and allow Fe(III) diffusion away from the cell (Hegler et al., 2010; Schädler et al., 2009). Finally, in the case of acidophilic FeOB, the main product is dissolved Fe^{3+} and Fe(III) minerals precipitate only as Fe^{3+} concentration increases and supersaturation is reached.

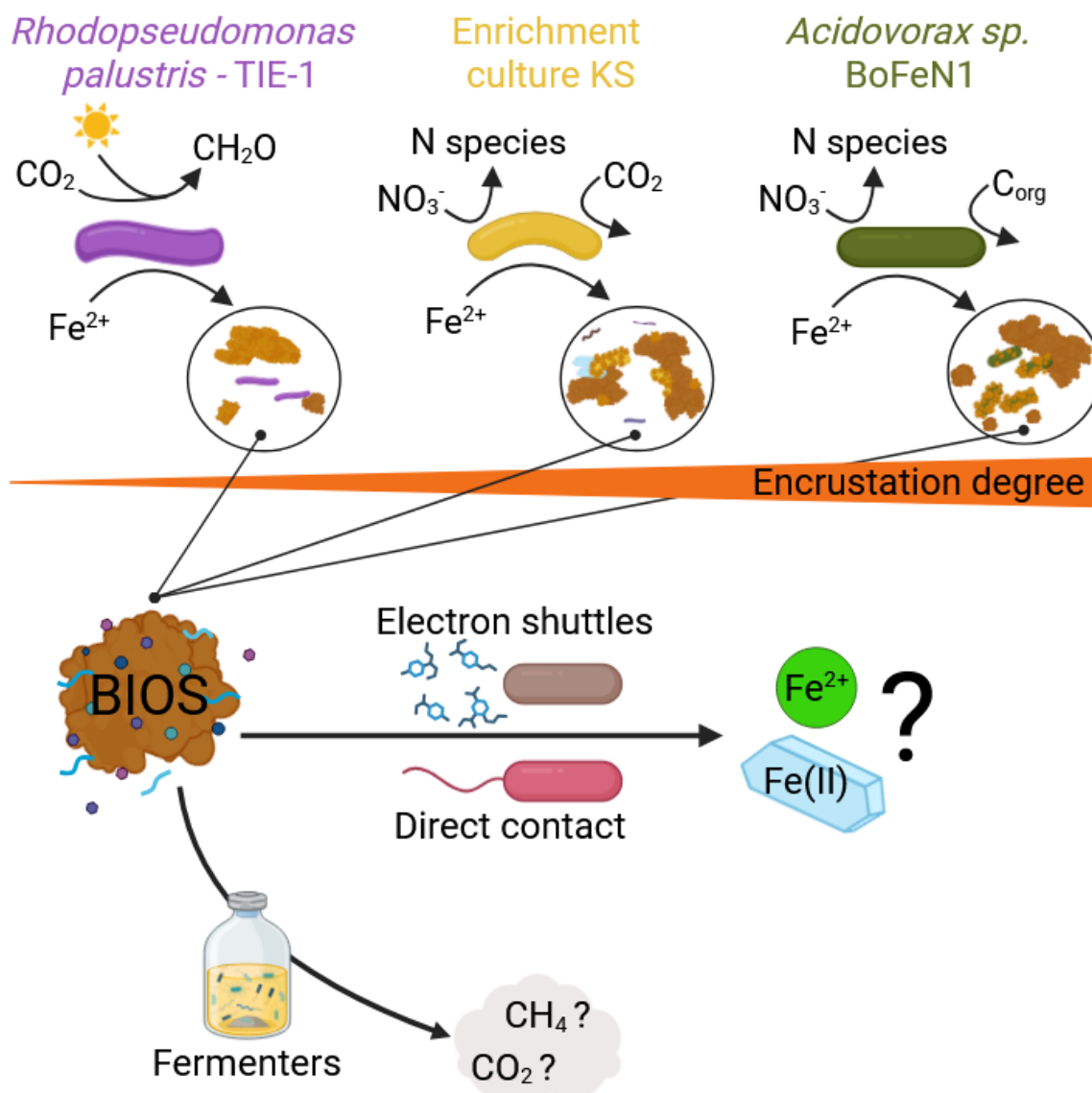


Figure 1. Fe(II)-oxidizing bacteria used in this work, their metabolism, a representation of the BIOS that they form, and subsequent transformation by FeRB and fermenters.

Biosignatures

Organic molecules preserved in the rock record and microfossils are often used as biosignatures, i.e. are evidence of past and present life (Eroglu et al., 2023; Des Marais et al., 2008; Javaux & Benzerara, 2009; Summons et al., 2008). The distinct properties of BIOS and their potential to preserve organic matter make them good biosignatures candidates (Chan et al., 2011, 2016; Hallberg, 2004). The BIOS of microaerophilic FeOM found in sediments from 1.7 Ga have been used as proof of this metabolism in the early Earth and to constrain the redox state of the atmosphere at that time (Chan et al., 2011; Slack et al., 2007; Little et al., 2004). This highlights the importance of understanding the formation of BIOS and their transformation under different conditions compared to abiotically formed Fe(III) minerals.

Cell encrustation encourages preservation of FeOB and other microbes in the community (Miot et al., 2015; Parenteau & Cady, 2010). Moreover, it can preserve the cell shape under long term diagenetic conditions (up to 20 h at 600°C or for 100 h at 300°C) (Li et al., 2013). However, BIOS are more bioavailable than abiotic ferrihydrite thanks to their higher surface area. In addition, the associated organic matter content may also act as a carbon source or electron donor (Langley, Gault, et al., 2009; Li et al., 2016; Roden, 2003; Whitaker et al., 2021; Ye et al., 2022). This ultimately transforms the minerals and degrades the biosignature.

Objectives

Biogenic iron oxides have intrinsic differences with their abiotic counterparts. They have been used as biosignatures in the form of microfossils in the rock record, and play an important role in the carbon cycle, acting as carbon sink. The main goal of this PhD thesis was to test the differences in their mineralogical properties, depending on the Fe(II)-oxidizing bacteria who produced them, and then test the susceptibility of this BIOS to further Fe reduction by Fe(III)-reducing bacteria and C degradation by fermentative bacteria.

Specifically, the objectives of this thesis were:

1) To determine the properties that influence the bioavailability of the Fe(III) associated with different BIOS for FeRB reduction (**Chapter 3**) by:

- a) Identifying the mineral phases in the BIOS produced by Fe(II)-oxidizing bacteria with different oxidation mechanisms.
- b) Determining the reduction rate and extent of the Fe(III) from the different BIOS.
- c) Correlating the reduction rate and extend to the availability of organic carbon.

2) To investigate the transformation and preservation potential of BIOS after microbial reduction (**Chapter 4**) by:

- a) Determining the mineral phase transformation after microbial Fe(III) reduction.
- b) Implementing high resolution microscopic techniques to determine the spatial distribution of cells within the minerals after reduction.
- c) Compare the cell preservation by different BIOS after reduction of the associated Fe(III).

3) To determine the reactivity of the BIOS-associated C to transformation by fermentative microbes (**Chapter 5**) by:

- a) Enriching fermentative microbes from the sediment of Lake Constance.
- b) Asses the BIOS-associated C transformation by fermentative microbes isolated with different carbon sources (yeast extract, autoclaved *S. oneidensis* and formaldehyde fixed *S. oneidensis*).

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Chapter 2. Authors contribution

Terrestrial iron biosignatures and their potential in solar system exploration for astrobiology

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The review was conceptualized by myself. M. Mansor and A. Kappler acquired the funding. Original draft of the manuscript was written by myself and reviewed by M. Mansor. All authors contributed to the review and editing of the final manuscript

Chapter 2. Terrestrial iron biosignatures and their potential in solar system exploration for astrobiology

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See glossary at the end of the article for explanations of specific terms.

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Abstract

Iron (Fe) is one of the most abundant elements in the solar system. It plays an important role in life by participating in redox reactions for energy generation (*e.g.*, by Fe(II)-oxidizing and Fe(III)-reducing microorganisms) and as a cofactor in multiple assimilatory metabolisms (*e.g.*, DNA replication). Fe-metabolizing microorganisms are ubiquitous on Earth, from soils and sediments to deep-sea hydrothermal vents. They catalyze Fe redox transformations between its most common redox species Fe(II) and Fe(III), and couple this to carbon degradation, CO₂ fixation, nitrate reduction and photosynthesis, thus linking the biogeochemical cycles of Fe with carbon and nitrogen. Biogenic Fe (oxyhydr)oxide minerals (BIOS), *i.e.* the products of neutrophilic Fe(II)-oxidizing microorganisms, are biosignatures of interest on Earth and with increasing interest on other habitable bodies in our solar system, such as Mars and icy moons. Here, we review the habitats, mechanisms, products and preservation of Fe-metabolizing microorganisms on Earth. We translate this knowledge into a biosignature context for the search of potential Fe-metabolizing microorganisms in the solar system.

Keywords: Iron-metabolizing microorganisms; Biosignatures; Early Earth; Mars; Icy moons

1. Introduction

Iron (Fe) is abundant in the solar system, especially in the rocky inner planets (Mercury, Venus, Earth and Mars). On Earth, Fe is the fourth most abundant element in the crust and the second most abundant redox-sensitive element, after oxygen. Although the majority of iron was sequestered in the core during accretion (McDonough, 2016), the remaining Fe in the crust, particularly Fe nanoparticles from hydrothermal and meteoritic origin could have acted as catalysts for the formation and complexification of organic compounds from the CO₂-rich early Earth atmosphere (Peters et al., 2023).

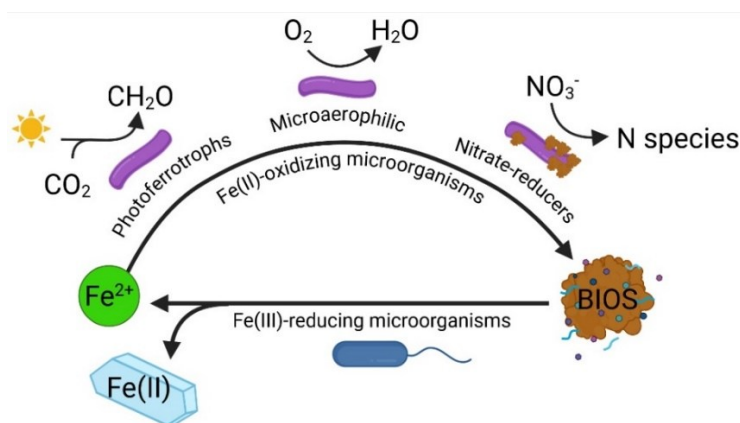


Figure 1. Overview of various microorganisms using Fe-based dissimilatory metabolisms that can produce, transform or degrade BIOS at neutral pH conditions.

Besides being an essential micronutrient in assimilatory metabolisms (with functions in DNA replication and gene expression), microorganisms can harvest energy from redox transformation between Fe(II) and Fe(III) with multiple mechanisms. For instance, at neutral pH conditions, Fe(II)-oxidizing microorganisms (FeOM) can use electrons from Fe(II) to fix CO₂ in photosynthesis (2.1 **Photoferrotrophs**), reduce O₂ (2.2 **Microaerophilic FeOM**) or reduce nitrate (2.3 Nitrate-reducing Fe(II)-oxidizing microorganisms (**NRFeOM**)), while others respire Fe(III) (2.5 **Fe(III)-Reducing Microorganisms (FeRM)**) (**Fig. 1**) (Kappler et al., 2021; Knoll et al., 2012; Konhauser et al., 2011), often in competition with abiotic processes. Neutrophilic FeOM produce various biogenic iron (oxyhydr)oxide minerals (BIOS) such as ferrihydrite ($(\text{Fe}^{3+})_{4-5}(\text{OH},\text{O})_{12}$), goethite ($\alpha\text{-Fe}^{3+}\text{O}(\text{OH})$), magnetite ($\text{Fe}^{2+}\text{Fe}^{3+}_2\text{O}_4$) and lepidocrocite ($\gamma\text{-Fe}^{3+}\text{O}(\text{OH})$) (Cornell & Schwertmann, 2006). Compared to abiotically formed Fe(III) (oxyhydr)oxides, the minerals within BIOS are usually poorly crystalline with larger surface areas due to their smaller crystal sizes which is primarily due to the presence of organic matter that blocks the surface from further growth (Vollrath et al., 2012; Zegeye et al., 2014). Moreover, they are intimately associated with organic matter such as

lipids, polysaccharides and even whole cells. This tight association leads to different properties, which is of interest for the utilization of BIOS as potential biosignatures.

Fe-based biosignatures are useful to understand the biological history of the Earth - ranging from the role of FeOM in the formation of Banded Iron Formations (BIFs) (2.8-2.5 Ga) (Chan et al., 2016; Croal et al., 2009; Klein, 2005; Posth et al., 2014; Posth, Konhauser, et al., 2013), Fe oxide filaments with organic matter in hydrothermal vent deposits from 4280 to 490 Ma (Dodd et al., 2017; Little et al., 2004) and spheroidal Fe(III) (oxyhydr)oxides in 100 Ka in Jurassic rocks (Potter-McIntyre et al., 2014). Using Earth as a case study, and with the knowledge that other bodies in the solar system are rich in Fe, Fe-based biosignatures can be promising in the search for life in these places.

The possibility for the presence of Fe-metabolizing bacteria has been raised on Mars, in particular regarding NRFeOM and phototropic FeOM (photoferrotrophs) (Price et al., 2018; Tabata et al., 2021). A handful of experiments and models have shown that these microorganisms can grow under Martian-like conditions (Bauermeister et al., 2014; Khuller et al., 2024; Palma et al., 2024; Silva et al., 2024). More recently, the possibility of Fe biogeochemistry on icy worlds has been proposed (Ray et al., 2021; Sahai et al., 2024). The minerals near the bottom of oceans in icy worlds likely contained reduced FeS and Fe-Ni (Zolotov & Shock, 2004a). Furthermore, the reaction between water and ultramafic rock around possible hydrothermal vents will generate Fe(II) on the seafloor (Běhouňková et al., 2021; Glein & Waite, 2020; Hsu et al., 2015). Hence, similar to around Earth's hydrothermal vents, dissolved iron could serve as electron source or precipitate as Fe minerals (e.g., goethite) (Emerson & Moyer, 2002). However, Fe minerals can be transformed or even destroyed by certain processes e.g., diagenesis, burial, and reduction by Fe(III)-reducing microorganisms (FeRM), opening questions about the preservation of BIOS under these conditions.

In this review, we briefly describe the different mineral phases of Fe on Earth and their role in dissimilatory Fe-based metabolisms (**Table 1**). Using this as a guideline, we then review the potential of Fe-based metabolisms beyond Earth, focusing on Mars and icy worlds. We end by highlighting Fe-based biosignatures that may exist in these environments as a guide to search for life in the solar system.

Table 1. Relevant Fe minerals in the context of biosignatures on Earth and Mars.

Mineral (formula)	Occurrence	Relevance
Ferrihydrite ($(\text{Fe}^{\text{III}})_{4-5}(\text{OH},\text{O})_{12}$)	Earth: major component of soils and sediments (Elias & Alderton, 2021). Mars: one of the main components of the Martian surface (Valantinas et al., 2025).	It is the most bioavailable Fe(III) (oxyhydr)oxide on Earth (Nixon et al., 2012), present in many BIOS (Langley, Igric, et al., 2009; Miot, Benzerara, Morin, et al., 2009).
Goethite ($\alpha\text{-Fe}^{\text{III}}\text{O}(\text{OH})$)	Earth: common component of soils and sediments (Van Der Zee et al., 2003). Mars: Gale crater (Klingelhöfer et al., 2005).	Earth: one of the biogenic minerals within BIOS. Mars: its occurrence in Gale crater supports the presence of water in early Mars (Klingelhöfer et al., 2005).
Hematite ($\alpha\text{-Fe}_2\text{O}_3$)	Earth: BIF deposits (Hagemann et al., 2016). Mars: it occurs as small spherules in the rocks at Meridiani planum (Squyres et al., 2004).	Mars: its formation on Mars can constrain the water budget and therefore add on the habitability of early Mars (Hynek et al., 2002; Squyres et al., 2004).
Magnetite ($\text{Fe}^{\text{II}}\text{Fe}^{\text{III}}_2\text{O}_4$)	Earth: found in soils, sediments and deposits such as the Atacama-Coquimbo Ferriferous Belt, Northern Chile (Espinoza, 1990) and BIF (Hagemann et al., 2016).	Earth: biogenic magnetite differs from abiogenic magnetite in their size, chemical purity and magnetic properties (Kappler et al., 2023; Pérez-Huerta et al., 2022). It has been used as biosignature for the presence of magnetotactic bacteria (e.g., by comparing the single-domain (SD) magnetic state and the uniaxial anisotropy of the magnetite chains) (Mathon et al., 2024; Petryshyn et al., 2016; Slotznick et al., 2023).
Siderite (FeCO_3)	Earth: sediments, hydrothermal environments (Ohmoto et al., 2004) and BIF (Konhauser et al., 2017).	Earth: one of the main byproducts of Fe(III) reduction. It plays a role in Fe and C biogeochemistry and C sequestration (Roh et al., 2003).
Vivianite ($\text{Fe}_3(\text{PO}_4)_2 \cdot 8\text{H}_2\text{O}$)	Earth: sediments, waterlogged soils, bogs, hydrothermal deposits and wastewater sludges (Rothe et al., 2016)	Earth: main byproduct of biogenic reduction of BIOS under controlled laboratory conditions (Langley et al., 2009).
Jarosite ($\text{KFe}^{\text{III}}_3(\text{SO}_4)_2(\text{OH})_6$)	Earth: oxidation of sulfide deposits, clay environments, acidic soils, mine tailings and one of the main components of the Iberian Pyrite Belt (IPB) (Dutrizac & Jambor, 2000). Mars: Meridiani Planum (Morris et. al 2004).	Mars: its presence in Meridiani Planum, Mars is proof of a wet, oxidizing and acidic environment on early Mars (Morris et. al 2004).

2. Earth

On Earth, Fe can be oxidized and reduced abiotically by various oxidants (O_2 , MnO_2 , NO_2^- , light) and reductants (sulfide, natural organic matter, light). In addition to these abiotic processes, microbial activities contribute significantly to the redox cycle of Fe due to their ubiquity on Earth and their rapid enzymatic kinetics (Kappler et al., 2021). In modern times, Fe(II), a soluble and bioavailable form for assimilatory metabolisms, is typically found in suboxic and anoxic environments such as wetlands, groundwater and paddy soils among others (Bhattacharyya et al., 2018; Huang et al., 2021). Fe(II) occurs as dissolved species in acidic environments where the low pH prevents its rapid oxidation or at circumneutral pH as Fe(II)-ligand complexes, where the associated organic matter alters its redox potential and stabilizes it against oxidation (Daugherty et al., 2017; Kappler et al., 2021). Finally, it also occurs as Fe(II)-containing minerals such as vivianite ($Fe_3(PO_4)_2 \cdot 8H_2O$), siderite ($FeCO_3$), Fe sulfides or clays (Pentáková et al., 2013; Mansor et al., 2025). However, Fe occurs predominantly in the form of Fe(III), which is poorly soluble at neutral pH and less bioavailable for assimilatory metabolisms, therefore requiring the production of siderophores to make it available by chelation (Ahmed & Holmström, 2014). Fe(III) is found mainly forming (oxyhydr)oxide minerals such as ferrihydrite (Fh), goethite and hematite (α - Fe_2O_3) (Pentáková et al., 2013).

BIOS are metabolic products of neutrophilic FeOM. BIOS are mixtures of Fe minerals, organic matter and cells (**Fig. 2**). The mineral portion of BIOS is typically composed of Fh, goethite and amorphous Fe(III) phosphates (Ferris, 2005; Langley et al., 2009; Miot et al., 2009). BIOS are highly mixed with organic matter and biopolymers, mainly lipid and polysaccharide moieties (Chan et al., 2009; Sowers et al., 2019; Whitaker et al., 2021). The outermost part of the BIOS is mainly composed of aliphatic groups associated with carboxylic moieties and acidic polysaccharides, while carboxylic and aromatic moieties are incorporated in the structure (Chan et al., 2009; Sowers et al., 2019). The Fe(III) minerals can also cover the cell membrane, leading to full or partial encrustation (Li et al., 2013; Miot et al., 2015). In fact, examples of exceptional preservation of cellular features associated with Fe(III) minerals are well known. Proteins from dinosaurs as old as 195-million years old were found to be preserved in hematite and goethite formed from Fe in Fe chelators (e.g., hemoglobin), which decreased the degradation of organic matter (Lee et al., 2017; Schweitzer et al., 2007). Thylakoid features detailing chloroplasts from phototrophic mats are preserved in modern hydrothermal iron deposits in Yellowstone National Park (Parenteau & Cady, 2010). Hence, Fe-rich BIOS may also preserve exceptional cellular features.

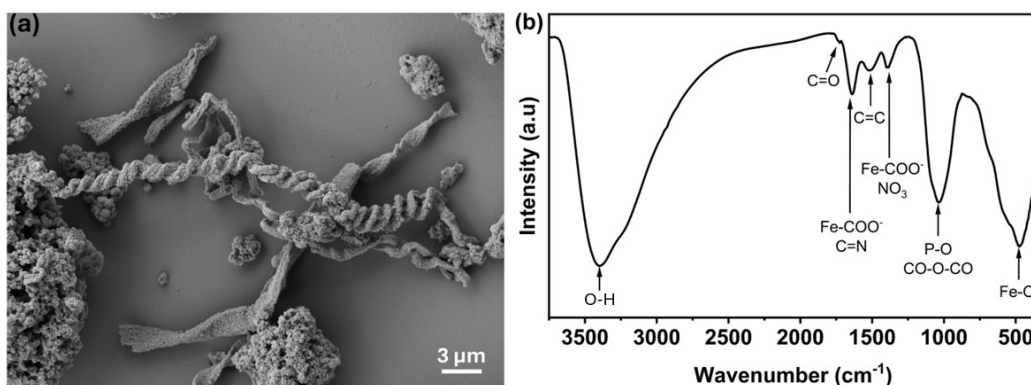


Figure 2. (a) Scanning electron micrograph of a sample from the iron-rich Segen Gottes mine (Black Forest, Germany). (b) Fourier transform infrared spectrum of BIOS from the nitrate-reducing Fe(II)-oxidizing strain *Acidovorax* sp. BoFeN1 grown in 22 mM bicarbonate-buffered medium and amended with 3 mM FeCl₂, 2 mM acetate and 6 mM nitrate, showing the signals from various organic functional groups.

BIOS can adopt intriguing morphologies that may be useful as biosignatures. In particular, micro-aerophilic FeOM (capable of growing with O₂ concentrations as low as 3 μM) can produce twisted stalks or sheaths (**Fig. 3**) (Chan et al., 2009, 2011; Krepski et al., 2013). These are extracellular organo-mineral structures mainly composed of poorly crystalline Fe(III) (oxyhydr)oxides and polysaccharides. They are produced to prevent cell encrustation and to position the cells at the optimal redox boundary (*i.e.*, where both O₂ and Fe(II) are present, by growing the stalk in the opposite direction of the cell movement) (Chan et al., 2009; Krepski et al., 2013). The organic matter associated with these biominerals alters the properties and reactivity of the Fe(III) minerals, leading to differentiation from abiogenic minerals (see section 2.6).

Naturally occurring poorly crystalline Fe(III) (oxyhydr)oxides are highly bioavailable for dissimilatory metabolisms (functioning as electron acceptor in anaerobic respiration), while with increasing crystallinity, their bioavailability decreases (Nixon et al., 2012). Under normal conditions, poorly crystalline Fe(III) (oxyhydr)oxides (*e.g.*, Fh) are more thermodynamically favorable for reduction than more crystalline phases. The relative bioavailability of different Fe(III) minerals can be determined by calculating their reduction potential (E_H), which can then be extended to the Gibbs free energy of reaction (ΔG_r). More negative ΔG_r implies higher bioavailability for reduction, which has been shown repeatedly for ferrihydrite over more crystalline Fe(III) minerals such as goethite and hematite (Kappler et al., 2021; Nixon et al., 2012; Schwerdtelm et al., 2025). However, as ΔG_r varies as a function of pH, temperature, pressure, substrate/reactant concentrations and particle

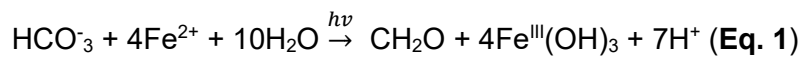
size (Amend & LaRowe, 2019; Kappler et al., 2021; Schwerdtelm et al., 2025), calculations should be performed under the environmental conditions of interests (e.g., Earth vs Mars vs icy moons) whenever possible.

Geobacter and *Shewanella* are two model FeRM typically used to investigate Fe(III) biogenic reduction on Earth. *Shewanella putrefaciens* CN-32, for instance, is capable of reducing hematite, although the reduction rate varies according to the size and facet. The smaller the particles the more bioavailable are both hematite and goethite (Xu et al., 2025). Hematite {001} is more bioavailable than hematite {100}, due to the different cell-mineral interactions and the higher conductivity of hematite {001} (Hu et al 2020), highlighting the importance of mineral characterization to understand controls on Fe(III) reduction rate. Fe(III) minerals within phyllosilicates (montmorillonite, nontronite, and illite) are also available for biogenic reduction (Jaisi et al., 2007; D. Liu et al., 2016; Y. Liu et al., 2023; Vodyanitskii, 2007). Remarkably, BIOS are more bioavailable than abiotic Fh, due to their higher surface area that provides more reactive sites per g of biomineral. The associated organic matter content may also act as a carbon source or facilitate electron transfer between the cells and the minerals (Langley, Gault, et al., 2009; Li et al., 2016; Roden, 2003; Ye et al., 2022). The origin of BIOS also affects their bioavailability. For example, BIOS from *Gallionella*-like FeOM were found to be less bioavailable for reduction by *Shewanella putrefaciens* CN-32 than those from *Leptothrix*-like FeOM (Langley et al., 2009).

In the next subsections we expand on the different Fe metabolisms, describing their occurrence in space and time. We highlight the BIOS that they produce, the transformations that they can undergo and their preservation potential.

2.1 Photoferrotrophs

Photoferrotrophs are a type of FeOM that can utilize sunlight energy and electrons from Fe(II) to fix CO₂ (**Eq. 1**) (Camacho et al., 2017; Widdel et al., 1993).



On modern Earth, they have been found in sediments and water bodies, where they prefer anoxic conditions and low light intensity (Hegler et al., 2008). The rock record indicates that the first photoferrotrophs appeared around ~3.5 Gyr ago, before oxygenic phototrophy (Gupta et al., 2021; Lyons et al., 2024; Xiong, 2006). As a result of Fe(II) oxidation, they form BIOS composed mainly of poorly crystalline minerals (e.g., Fh) and goethite (Jiao et al., 2005). Due to the negative charge

of the cells, positively charged iron ions can be sorbed to the cell wall, leading to the precipitation of minerals on the cell membrane. This cell-mineral association can protect cells from dangerous UV radiation (Gauger et al., 2015), although it can also interfere with photosynthesis (Wu et al., 2014). Therefore, some species of photoferrotrophs have developed mechanisms to avoid this, such as by modifying its surface composition to cause an electrostatic repulsion between the cell and the minerals (Stukalov et al., 2008; Thompson et al., 2019). They may also produce negatively charged extracellular polymeric substances (EPS) that complex the Fe(III) produced during oxidation, preventing its attachment to the cell surface (Chan et al., 2011; Wu et al., 2014). In addition, they can acidify the surrounding microenvironment to keep the Fe(III) dissolved and allow Fe(III) diffusion away from the cell (Hegler et al., 2010; Schädler et al., 2009).

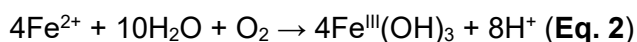
The early appearance of photoferrotrophy and the lack of encrustation suggest that these bacteria might have played a role in the formation of BIFs (Kappler et al., 2005). BIFs are extensive deposits of Fe(III) (oxyhydr)oxides (mainly magnetite and hematite) and silica that were enigmatically formed already on early Earth when atmospheric O₂ was low (and later when O₂ was formed by cyanobacteria to a larger extent). The mechanism of deposition of the oldest BIFs (i.e. the identity of the Fe(II) oxidation mechanism that lead to Fe(III) mineral precipitation) is debated. Several mechanisms were proposed including ultraviolet (abiotic) photooxidation, direct enzymatic Fe(II) oxidation by anoxygenic photoferrotrophs, chemical oxidation by traces of O₂ produced by the earliest cyanobacteria, enzymatic oxidation by microaerophilic Fe(II)-oxidizers using the traces of O₂ produced by the earliest cyanobacteria, or a combination of these processes (Dodd et al., 2022; Dreher et al., 2021; Thompson et al., 2019; Konhauser et al., 2007).

Photoferrotrophic Fe(II) oxidation likely played a role for the deposition of the oldest BIFs given (a) the suitable conditions for photoferrotrophy in the ancient ocean (high Fe(II) and low O₂ in the photic zone) (Kappler 2005) (b) lack of organic matter in BIFs, explainable by low association between photoferrotrophs and the Fe(III) minerals that they formed (Posth et al., 2010; Thompson et al., 2019), (c) consistency between BIF deposition rate (1.4 mol m⁻² yr⁻¹) (Canfield et al., 2006) with those calculated for model photoferrotrophs (Kappler 2005) and measured in modern ferruginous lakes (3.4 mol C m⁻² yr⁻¹) (Llirós et al., 2015). The roles of abiotic ultraviolet photooxidation and oxidation via O₂ produced by the earliest cyanobacteria cannot be fully ruled out, and ongoing research are discovering how interactions between Fe(II), silica, reactive oxygen species and Fe(III) reducing bacteria can affect Fe(II) oxidation rate and extent and the identity of the precipitated minerals (Dreher et al., 2025; Gauger et al., 2016; Huang et al., 2021; Schad et al., 2022;

Swanner et al., 2015; Yang et al., 2024). After precipitation and sedimentation, diagenesis would transform ferrihydrite (main component of BIOS), that may have been the initial mineral phase, into hematite and magnetite, the main components of BIFs today (Konhauser et al., 2023). Additionally, should organic carbon (OC) precipitate with the Fe(III) minerals, long-term diagenesis and metamorphism would degrade the OC and produce Fe(II)-bearing minerals (Halama et al., 2016; Köhler et al., 2013; Posth et al., 2013). Hence, preservation of direct biosignatures in BIF deposits is still an open question.

2.2 Microaerophilic FeOM

Increasing O₂ concentration in the atmosphere after the Great Oxidation Event led to the expansion of habitats suitable for the proliferation of microaerophilic FeOM. Microaerophilic FeOM use Fe(II) as electron donor to reduce O₂ (**Eq. 2**) in circumneutral pH environments (Hedrich et al., 2011).



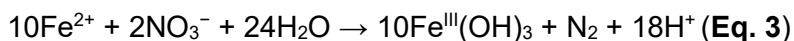
These microorganisms are generally found in environments where the concentration of O₂ is between 3 and 50 µM that O₂ allows them to outcompete abiotic oxidation (Druschel et al., 2008; Lueder et al., 2022; Maisch et al., 2019). Microaerophilic FeOM (e.g., *Gallionella ferruginea*, *Mariprofundus ferrooxydans*) produce various BIOS typically comprising of poorly crystalline Fh, lepidocrocite, goethite or akageneite (Hallbeck & Pedersen, 1990; Krepski et al., 2013; Laufer et al., 2017). These BIOS can adopt unique morphologies such as twisted stalks, tubular sheaths, granular or dreadlock-like structures (**Fig. 3**) (Chan et al., 2011, 2016; Hallberg, 2004). The morphology of these BIOS is highly dependent on the niche and the FeOM. For instance, BIOS of *Gallionella*, an autotrophic microaerophilic FeOM that preferentially occurs in low-OC environments can be in the form of twisted stalks, while *Leptothrix*, an heterotrophic FeOM that is typically found in high-OC environments can produce sheaths (Tothero et al., 2024). Moreover, the formation of these structures is regulated at the gene level by specific genes such as the xag operon, associated to stalk-formation (Kato et al., 2015). These structures are originally EPS secreted by the microorganisms, which then become a template for Fe(III) mineral nucleation and precipitation. The EPS also differs among species (Chan et al., 2004; Miot, Benzerara, Morin, et al., 2009), but the details are unknown for most FeOM (Makita, 2018). This strategy allows them to avoid cell wall encrustation by Fe(III) minerals that would hinder their metabolic activities.

Some microaerophilic FeOM are able to live in environments with higher concentrations of O₂. For example, Baker et al., (2023) demonstrated that *Ghiorsea bivora* TAG-1 excretes exometabolites that increase the half-life of Fe²⁺ by two orders of magnitude (from 3.3 to 335.5 days) in oxic seawater. The BIOS of *G. bivora* TAG-1 also differed from abiogenic Fe(III) (oxyhydr)oxides. This BIOS consisted of small particles (~100–500 nm) aggregated in a globular shape mainly composed of poorly crystalline phases (Fh-like), while in the abiotic control, the Fe(III) (oxyhydr)oxides were needle-like and bigger (~1 µm in length) composed of more ordered phases (lepidocrocite, akaganeite, goethite, feroxyhyte) (Baker et al., 2023).

These special morphologies of the BIOS formed by microaerophilic FeOM have been used as biosignatures of Fe metabolism in sediments from 1.7 Ga (Slack et al., 2007; Little et al., 2004). They also provide information about the redox state of the atmosphere of the early Earth, given that their formation relies on the presence of O₂ (Chan et al., 2011). Interpreting these structures in the rock record requires caution, as abiotic processes can form Fe(III) minerals with BIOS-like shapes, leading to false positives (Criouet et al., 2021; McMahon, 2019; Cosmidis & Templeton, 2016). This highlights the importance of a profound understanding of BIOS, their formation and transformation under different conditions and their unique morphology and composition (cell-mineral associations) compared to false positives in the investigation of Fe-based biosignatures (see section 2.6).

2.3 Nitrate-reducing Fe(II)-oxidizing microorganisms (NRFeOM)

NRFeOM harvest electrons from Fe(II) for the reduction of nitrate (NO₃⁻) to gain energy (**Eq. 3**).

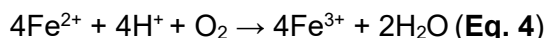


After the radiation of oxygenic photosynthesis, the high oxygen concentration allowed for the expansion of the nitrogen cycle (Stüeken et al., 2016), paving the way for direct coupling between the nitrogen and iron biogeochemical cycles. NRFeOM can either fix CO₂ (autotrophic) or use a bioavailable carbon source (e.g., acetate) as a co-substrate (mixotrophic) (Dopffel et al., 2022; Huang et al., 2021; Tominski et al., 2018). NRFeOM produce various nitrogen intermediates that follow a sequential reduction: NO₃⁻ → NO₂⁻ → NO → N₂O → N₂ (Jakus et al., 2021). Nitrite and nitric oxide rapidly oxidize Fe(II) abiotically (Klueglein et al., 2014), which may play a crucial role in the oceanic primary production by limiting the sedimentary Fe supply to the surface (Scholz et al., 2016).

NrFeOM have been isolated from modern freshwater sediments, groundwater and soils (Liu et al., 2019; Straub et al., 1996). They cohabit with phototrophs and microaerophilic FeOM in the sediments and in water columns (Laufer et al., 2016; Otte et al., 2018). However, it has been found that reactive nitrogen species (e.g., NO) byproducts of NrFeOM are toxic to photoferrotrophs (Nikeleit et al., 2024). This raises questions on microbial interactions and competition for Fe as an energy source, particularly on early Earth. Contrary to phototrophic and microaerophilic FeOM, some NrFeOM can become encrusted (with exception of those autotrophs) and entombed by the residual Fe(III) that precipitates in the cell membrane due to attraction to the negatively charged membrane (Miot et al., 2009). To some extent, this encrustation could be beneficial, preventing cell dehydration, but it can also prevent the uptake of nutrients leading to cell death (Knoll et al., 2012). This encrustation is of geological importance because it increases the preservation potential of the cells in the rock record, not only of FeOM but also of other microbes in the community (Miot et al., 2015; Parenteau & Cady, 2010). BIOS preserve protein globules and peptidoglycan such as those in the periplasm of *Acidovorax* sp. strain BoFeN1 (Miot et al., 2011) and can preserve the cell shape completely under diagenetic conditions (up to 20 h at 600°C or for 100 h at 300°C) (Li et al., 2013).

2.4 Acidophilic FeOM

Under acidic conditions (pH <4), Fe(II) and oxygen are stable together in solution (Stumm and Morgan, 1981). The redox potential of the ferrous/ferric iron couple (+770 mV at pH 2) makes oxygen the most suitable electron acceptor for acidophilic FeOM (Eq. 4).



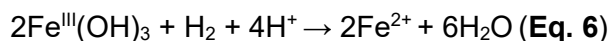
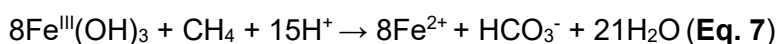
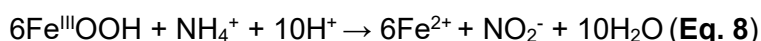
Hence, the isolates of acidophilic FeOM are obligate aerobes (Johnson & Hallberg, 2008; Bird et al., 2011; Johnson, 1998). To the best of our knowledge, no acidophilic photoferrotrophs or NrFeOM have been described so far. Acidophilic FeOM differ in their affinity for Fe. Bacteria of the genus *Leptospirillum* are expected where the concentration of Fe(III) is high, while *Acidithiobacillus ferrooxidans* is dominant where Fe(II) is depleted and pH is low (<3) (Johnson, 1998; Jones et al., 2015).

The main product of acidophilic FeOM is dissolved Fe³⁺. No direct BIOS formation can be attributed to them. Nonetheless, as Fe³⁺ concentration increases, Fe(III) minerals precipitate once supersaturation is reached. Indirect Fe(III) minerals produced include schwertmannite and jarosite, that later transform into goethite in aqueous environments at pH ≥ 4 (Acero et al., 2006;

Bigham et al., 1996). The formation of schwertmannite or jarosite will depend on the presence of cations (K^+ , NH_4^+ , Na^+ and H^+) and the pH. Jarosite is favored at high cations concentration and low pH (Jones et al., 2014). No particular biogenic morphology is attributed to these Fe(III) minerals, although EPS produced by acidophilic FeOM can facilitate their precipitation (Kaksonen et al., 2014). These minerals can help in the preservation of molecular biosignatures that would otherwise degrade in acidic environments (Souza-Egipsy et al., 2010). Goethite is particularly known to aid in lipid preservation by strongly binding to carboxylic and hydroxyl functional groups, preventing exposure of their reactive groups (Filius et al., 2000; Kaiser & Guggenberger, 2007; Tan et al., 2018). Lipids are commonly used as molecular biosignatures because as opposed to other biomolecules, they preserve their hydrocarbon skeleton for thousands of millions of years (Finkel et al., 2023). Hence, despite the lack of BIOS, the Fe^{3+} produced by acidophiles can help in the preservation of molecular biosignatures.

2.5 Fe(III)-Reducing Microorganisms (FeRM)

To complete the biogeochemical cycle of Fe, we discuss microbial respiration of Fe(III) performed by Fe(III)-reducing bacteria (FeRM). Respiration of Fe(III) is an ancient metabolism (~3.5 Gyr) (Lyons et al., 2024). FeRM are found in marine and freshwater sediments, where they reduce Fe(III) to Fe(II) coupled to the oxidation of organic carbon, H_2 , CH_4 or ammonia (Eq. 5, 6, 7, 8).



They are capable of oxidizing various organic molecules such as acetate, lactate and even complex aromatic compounds (Wang et al., 2008). To date, both neutrophilic and acidophilic FeRM have been described. Acidophilic FeRM, first described in 1976 (Brock & Gustafson, 1976), can use H_2 , sulfur and organic compounds as electron donors and are part of the phyla Crenarchaeota, Euryarchaeota, Acidobacteria, Actinobacteria, Firmicutes, and Proteobacteria (Malik & Hedrich, 2022). FeRM can reduce a variety of Fe(III) minerals (e.g., Fh and hematite) with varying rate and extent depending on the physical properties of the minerals, forming (mixed valent) Fe(II) minerals such as siderite, vivianite, magnetite or green rust ($[Fe(II)_{1-x}Fe(III)_x(OH)_2]^{x+}[(x/n)A^{n-}, mH_2O]^{x-}$, with A^{n-} : intercalated anions and x: molar fraction of trivalent cations).

Fe(III) is much less soluble than Fe(II). Most organisms depend on the production of siderophores for its assimilation (Ahmed & Holmström, 2014). However, FeRM have developed different reduction strategies to overcome this challenge. They use membrane proteins (c-type cytochrome) in the cell surface to reduce Fe(III) in direct cell-mineral contact (Shi et al., 2009). They can use pili or nanowires to transport electrons to the electron acceptor, increasing the required cell-mineral distance from nm to few μm (Reguera et al., 2006; Smith et al., 2013). Another strategy involves electron shuttles that are reduced enzymatically, with the electrons transferred to the final acceptor abiotically (Bai et al., 2020). Electron shuttles allow the electron transfer to occur in a cm-scale and can be found in the environment (e.g., natural organic matter) or be secreted by microorganisms (e.g., flavins) (Bai et al., 2020).

It is important to clarify that FeRM can use both the mineral and organic components of BIOS as substrates regardless of the different origin and properties (Whitaker et al., 2021). The mineral component of BIOS can be used as electron acceptor. The organic matter associated to BIOS can be used as electron donor. Additionally, the organic component can stabilize poorly crystalline Fe(III) minerals by preventing Fe(II)-catalyzed recrystallization (Kennedy et al., 2004; Warren & Ferris, 1998). This leads to a higher rate and extent of BIOS reduction compared to abiogenic iron (oxyhydr)oxides (Langley et al., 2009). Hence, the preservation of BIOS as biosignatures also relies on the biogenic processes that affect their fate after initial formation.

2.6 Biosignatures

2.6.1 Morphological biosignatures

Biosignatures are evidence of past and present life. They may include molecules (e.g., lipids), minerals (e.g., BIOS, microfossils) or specific patterns (e.g., isotopic, microbialites) (Eroglu et al., 2023; Des Marais et al., 2008; Javaux & Benzerara, 2009; Summons et al., 2008). Their correct interpretation is crucial to understand the history of life on Earth and in the search of life elsewhere. BIOS produced by FeOM are prime candidates for Fe-based biosignatures. BIOS are typically poorly crystalline and with an irregular morphology (e.g., minerals associated with twisted stalks and sheaths, **Fig 3**) (Chan et al., 2004, 2009, 2011; Cornell & Schwertmann, 2006). They have a higher surface area, mainly due to the small particle size (Eusterhues et al., 2008; Kappler et al., 2005). They also have a higher organic carbon content, mainly from EPS and entire cells entombed by the Fe(III) (oxyhydr)oxides (Chan et al., 2009; Miot, Benzerara, Morin, et al., 2009; Miot, Benzerara, Obst, et al., 2009). Some BIOS also precipitate on the cells, leading to the preservation of their morphology (*i.e.*, microfossils) (Williams et al., 2015) and associated organic

matter. FeOM have unique coenzymes that can be preserved in the rock record (Floyd et al., 2019) and BIOS can also help in the preservation of biomolecules with different origin (e.g., Scytonemin, a cyanobacterial pigment) (Varnali & Edwards, 2013).

The morphological analysis of these structures is performed by microscopic techniques, utilizing a combination of light, scanning and transmission electron microscopy (**Fig. 3**) (Chan et al., 2011; Suzuki et al., 2012).leading to The organic composition of the BIOS is studied mainly by spectroscopic techniques (e.g., X-ray absorption spectroscopy and FTIR) and secondary ion mass spectrometry for the EPS composition (Chan et al., 2016; Miot, Benzerara, Obst, et al., 2009; Suzuki et al., 2012). Moreover, fluorescence microscopy and sequencing are used to elucidate the distribution and species of the cells (or cell remnants) associated with the minerals (Fleming et al., 2014; Krepski et al., 2013; Suzuki et al., 2012). Confocal fluorescence microscopy is in particular useful to identify the distribution pattern of polysaccharides and cells with respect to the Fe minerals (Krepski et al., 2012, 2013; Laufer et al., 2017).

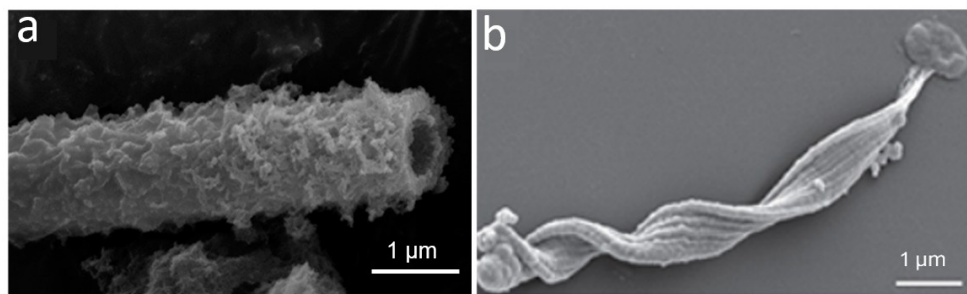


Figure 3. Different morphologies of BIOS. a. Scanning electron microscopy (SEM) image of *Lep-tothrix* spp. sheath coated with Fe(III) (oxyhydr)oxides (Suzuki et al., 2015) b. Field emission SEM image of a *M. ferrooxydans* PV-1 stalk (Chan et al., 2016).

However, the interpretation of Fe(III) (oxyhydr)oxides minerals for biogenicity is not trivial. Abiotic reactions can form so-called biomorphs, biogenic-like structures that resemble the morphology of microfossils and can lead to misinterpretation. For instance, cell-like structures can be formed by S^0 encapsulated in organic matter that self-assembled in a sulfidic environment (Cosmidis & Templeton, 2016), by diagenesis (200°C, ~15 bars) of quartz in the presence of RNA (Criouet et al., 2021) and by chemical gardens (biomorphs formed by the reaction of a ‘seed’ metal salt into an aqueous silicate, phosphate, carbonate, oxalate, or sulfide solution) (McMahon, 2019). In addition, in some conditions abiotic biomorphs can be better preserved than their biogenic

counterparts (Nims et al., 2021). Therefore, multiple approaches (e.g., morphologic, isotopic, organic and mineral composition) must be followed to assess the biogenicity of a sample.

2.6.2 Isotopic biosignatures

While Fe isotopes were thought to be initially promising for biosignatures, multiple studies have concluded that the isotopic fractionations associated with biogenic versus abiogenic Fe(II) oxidation and subsequent precipitation showed significant overlaps (Johnson et al., 2020). This is further compounded by Fe isotopic exchange between minerals and aqueous phase during burial and diagenesis (Severmann et al., 2006). In contrast, carbon isotopes seem more promising. High negative $\delta^{13}\text{C}$ values ($<-10\text{‰}$) of C associated to Fe(III) (oxyhydr)oxides are correlated with a biogenic origin (BIOS), while those of an abiotic origin typically have $\delta^{13}\text{C}$ values $>-10\text{‰}$ (Ayupova et al., 2016; Kennedy et al., 2010; K. A. Weber et al., 2012).

2.6.3 Transformation of BIOS via short term diagenesis

Short term diagenesis of BIOS is carried out by FeRM. The rock record and phylogenetic studies set the appearance of FeRM contemporary to photoferrotrophy (~ 3.5 Gyr ago) (Lyons et al., 2024). There are significant differences in the reduction rate, depending on the FeOM that produced the BIOS. For instance, Fe(III) in BIOS from *Gallionella*-like iron(II)-oxidizers were less bioavailable for reduction by *Shewanella putrefaciens* CN32 than those from *Leptothrix*-like iron(II)-oxidizers (Langley et al., 2009). In addition, organic matter embedded in the BIOS does not seem to inhibit the Fe(III) reduction, but to promote it, as it can be used as a nutrient source (Langley et al., 2009). FeRM and fermenters could have been responsible for up to 70% of Fe(III) reduction in ancient oceans (Konhauser et al., 2005), hence destroying or altering BIOS biosignatures.

Reduction of Fe(III) (oxyhydr)oxides by FeRM produces Fe^{2+} and leads to different mineralogy. The reduction of abiotic minerals forms Fe(II) minerals such as siderite and vivianite (Weber et al., 2006), the mixed-valent Fe(II)-Fe(III) minerals magnetite and green rust (Lovley, 1991; O'Loughlin et al., 2007), and the Fe(III) mineral goethite from dissolution-reprecipitation of poorly crystalline Fh catalyzed by Fe^{2+} (Hansel et al., 2004; Notini et al., 2022). When using BIOS, magnetite (Glodowska et al., 2021), vivianite and other hydrated ferrous phosphates of varying stoichiometries are formed (Langley et al., 2009). However, little is known about the effect of multiple variables in the reduction products of BIOS, such as the origin of the BIOS, the medium composition, the pH and Fe(III) concentration. One study showed that organic matter can be preserved

within biogenic siderite formed by microbial Fe(III) reduction (Han et al., 2024). Hence, FeRM may not completely erase biosignatures, but may preserve them in a different form instead.

Besides FeRM, BIOS can also be reduced abiotically by sulfide, generating a variety of iron sulfide minerals such as mackinawite (FeS), greigite (Fe₃S₄) and pyrite (FeS₂) (Mansor et al., 2025). The relative contribution of FeRM to other reductants depends on environmental parameters such as pH, sulfate concentration and microbial abundance. In this review, we focus on BIOS transformation under low sulfur conditions, although the impact of sulfur on BIOS preservation should be explored in future studies.

2.6.4 Long term diagenesis

Given enough time, temperature and pressure, the initial BIOS can be further transformed during burial, diagenesis and metamorphism. In aqueous environments, poorly crystalline Fe minerals transform abiotically into lepidocrocite, goethite and hematite, depending on pH, the presence of co-existing substances (e.g., Al, trace metals, organic matter), temperature, pressure and time (Caraballo et al., 2022; Posth et al., 2010). The preservation of Fh is known to up to 80°C due to its stabilization by organic matter (Kennedy et al., 2004; Toner et al., 2012). At higher temperatures and pressure, Fh transforms to siderite (100°C, 1-30 MPa) and magnetite (120°C, 1-50 MPa) in the presence of organic matter (Halama et al., 2016; Kennedy et al., 2004; Köhler et al., 2013; Posth et al., 2013). Meanwhile, hematite is the most thermodynamically stable Fe oxide and the main diagenetic product over geological timescales under oxidizing conditions (Cornell and Schwertmann 2003). Transformation of other Fe(III) minerals to hematite is facilitated at high temperatures, pressures and mechanical stress (Cornell and Schwertmann 2003). Nevertheless, goethite is occasionally found in the geological record, where their transformation to hematite could be suppressed under cool environments with acidic pH (Sephton et al., 2023). Additionally, under reducing and sulfidic conditions, the formation of pyrite is favored (Berner, 1984; Mansor et al., 2025; Raiswell & Canfield, 1998). Notably, Fe minerals play a key role in the preservation of organic matter in sediments, preserving them on 100,000 years' time scales (Klueglein et al., 2014), with the oldest report being a 3.770 Gy old microfossil of hematite with the morphology of the sheaths produced by FeOM, found in the Nuvvuagittuq Supracrustal Belt (NSB), Quebec (Dodd et al., 2017).

BIOS-Fe(III) is subjected to reduction in the presence of organic matter at high pressures and temperatures (Wu et al., 2014). However, BIOS can help in the preservation of organic matter.

BIOS of NRFeOM (e.g., *Acidovorax* sp. BoFeN1) help to preserve the cell content up to 600°C (Li et al., 2013). Picard et al. (2015) investigated the effect of diagenetic process (high T and/or P) on twisted stalks and found preservation of their morphology and organic content (up to 250°C/140 MPa), even though there is transformation into more crystalline minerals (e.g., hematite and magnetite). They concluded that such structures could be preserved in the rock record and be indicators of the presence of FeOM. Moreover, the released Fe³⁺ can precipitate onto other cells beyond the FeOM, thus helping in their fossilization and preservation (Orange et al. 2011; Ferris et al. 1988) although likely with a reduced efficiency (Beveridge et al., 1983; Picard et al., 2015).

In summary, Fe-metabolizing microorganisms play an important role in the biogeochemical cycle of Fe and other elements on modern and ancient Earth. Various FeOM produce BIOS that give clues to microbial communities and environmental conditions (e.g., O₂ concentration), despite multiple transformation processes after burial in the geological record. Therefore, BIOS are ideal candidates for the search of Fe biosignatures in other Fe-rich environments such as in other rocky planets and icy moons.

3. Mars

Mars, as part of the terrestrial planets, possesses an interior rich in iron (Fe) and nickel, and a silicate mantle and crust. Despite Fe being slightly less abundant in the bulk Mars (23.7 wt% (Yoshizaki & McDonough, 2020)) compared to Earth (32 wt%, McDonough 1995), Fe is preferentially partitioned in the upper Martian crust (11.4 wt% in ~1550 km thick layer; 4th most abundant) (Kim et al., 2023; Knapmeyer-Endrun et al., 2021; Yoshizaki & McDonough, 2020) with a value approximately twice than in Earth's crust (6.3% in ~2900 km thick layer) (Helffrich & Wood, 2001; McDonough & Sun, 1995), highlighting its availability for microbial metabolisms. Multiple rovers have investigated the planet starting with the Viking landers in 1976. The Mars Exploration Rovers equipped with a Mössbauer spectrometer identified various Fe-bearing minerals and clays including olivine ((Mg,Fe^{II})₂SiO₄), pyroxene (XY(Si,Al)₂O₆, with X: Ca, Na, Fe(II) or Mg; and Y: Cr, Al, Mg, Co, Mn, Sc, Ti, V, Fe(II) or Fe(III)), ilmenite (Fe^{II}TiO₃), magnetite, nanophase ferric oxide (npOx), hematite, goethite, and Fe^{III}-sulfates in Gusev crater (Morris et al., 2006); and hematite, jarosite (KFe^{III}₃(SO₄)₂(OH)₆), olivine and magnetite in Meridiani Planum (Christensen et al., 2000; Hoefen et al., 2003; Klingelhöfer et al., 2005; Mustard et al., 2005; Squyres et al., 2006; The Omega Team et al., 2005).

Today there are two operational rovers at the surface: The Curiosity rover from the Mars Science Laboratory mission and the Perseverance rover (Mars 2020). The Alpha Particle X-ray Spectrometer (APXS) onboard Curiosity revealed that the sedimentary rocks are rich in Fe with Fe content (as FeO) from ~20% to as high as 27.6%, most of it in the form of magnetite and poorly diffracting (oxyhydr)oxides (Treiman et al., 2016). With the CheMin X-ray diffraction (XRD) and X-ray fluorescence (XRF) instrument (Blake et al., 2012), Treiman et al., (2016) reported iron phases that suggest the presence of Fh which has recently been confirmed by infrared spectra and kinetic calculations (Valantinas et al., 2025). Given that Fh is the most thermodynamically bioavailable of the ferric iron (oxyhydr)oxides (Nixon et al., 2012; Cornell and Schwertmann 2003), its presence emphasizes the habitability of Mars and the potential for microbial Fe(III) reduction. It opens questions about the possible abundance or even presence of FeOM and FeRM. If Fh is so abundant, was any or all of it produced by FeOM? Is it being reduced or was part of it reduced at a given moment by FeRM?

Despite this ever-present Fe, the Martian surface is not considered habitable today due to the high UV radiation and the high concentration of strong oxidizing agents such as perchlorates and chlorates (Glavin et al., 2013; Hecht et al., 2009; Sutter et al., 2017). On Earth, perchlorates are found at low concentrations, except in arid regions such as in the Atacama Desert, where it can reach 0.1 wt% (Duncan et al., 2005; Srinivasan & Viraraghavan, 2009). On Mars, perchlorate concentration can reach up to 1 wt% (Rzymiski et al., 2024). However, the Martian subsurface could be a good habitat for life, given that it is warmer than the surface, it is protected from radiation and the potential presence of water reservoirs (Clark et al., 2021; Longo & Damer, 2020). Conditions conducive to life would have also existed on the surface of early Mars given the existence of a reducing atmosphere, the presence of water and organics (Westall et al., 2013; Amador and Ehlmann 2020). Hydrated minerals such as jarosite found at Meridiani Planum, the landing site of the Opportunity rover (Morris et al 2004), supports the presence of large amounts of water in the past during the Noachian era (~4.1-3.7 Gyr ago) (Marion et al., 2008; Moores et al., 2019). Organic matter would have been supplied by exogenous sources, potentially exceeding the concentration reached on early Earth (Clark et al., 2021). The presence of an atmosphere and reducing gases would have reduced the toxicity of reactive oxygen species compared to the modern day (He et al., 2023). In this section, we discuss the habitability of early and modern Mars, reviewing studies that test this hypothesis and identifying potential future research on iron-based dissimilatory metabolism and other metabolic processes that the planet could have supported or may still support.

3.1 Life based on Fe on early Mars

Early Mars had a favorable environment where protocells could have been formed (Cary et al., 2024; Clark et al., 2021). In the hypothesis that life originated, the availability of Fe would have been a high selective pressure that shaped the metabolism while catalyzing multiple reactions. Early Mars had a reducing atmosphere rich in N_2/CO_2 ~4.3 Gyr ago (Sasselov et al., 2020). Given the reducing conditions and high amounts of Fe (Yoshizaki & McDonough, 2020) and sulfur (S), these elements might have played an important role in the origin and development of life, although we will focus on Fe here. Fe is among the pool of redox-active species present on Mars (besides hydrogen, carbon, sulfur) that can act as both electron donor and acceptor due to its multiple valences.

In addition to an electron donor and acceptor couple, life (as we know it) requires carbon to build biomass and in some cases even uses organic carbon as substrate (i.e., as energy source). In Gale Crater, the discovery of OC raised an interesting question as to its ultimate source. A few studies considered whether the carbon could originate biologically from the activity of NRFeOM. Considering the availability of Fe(II) (Hurowitz et al., 2010), nitrate (Stern et al., 2015), organics (Eigenbrode et al., 2018), the circumneutral pH and the presence of liquid water in the Gale Crater, Price et al. (2018) proposed a plausible chemolithotrophic metabolism involving NRFeOM. As their metabolism does not require light, they could thrive deeper underground when the surface conditions turned hostile.

Fifer & Wong (2024) went further and estimated the expected concentration of autotrophic NRFeOM that would be needed to explain the amount of organic carbon in Gale Crater, in terms of preserved biomass (biomass per unit volume). Nitrate concentration is known (<1,100 ppm) (Stern et al., 2015) and carbon input from other sources (e.g., hydrothermal or atmospheric production) is expected to account for only 61 ppb (20-87% of the total). Assuming low consumption of nitrate (<10%), they calculated that $\sim 10^4$ cells/mL of NRFeOM could be expected in Gale crater lake in early Mars (**Fig. 4**). Considering that the global reservoir of nitrate on Mars is 5×10^{15} mol (Manning et al., 2009), and that organic carbon is continuously supplied today at a rate of $\sim 2.4 \times 10^5$ kg year⁻¹ endogenously or via meteorites (Yen et al., 2006), NRFeOM could be widespread on Mars. Caution is warranted, however, as nitrate and organic carbon concentrations may vary widely on the Martian surface. Moreover, this model did not include heterotrophs, although their presence cannot be excluded (Raven et al., 2018).

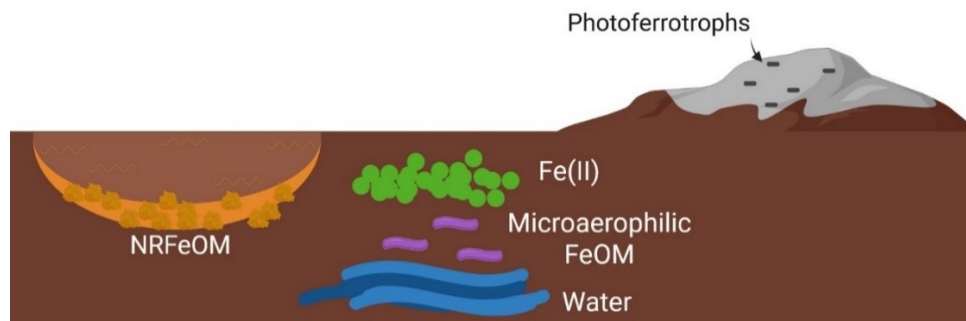


Figure 4. Potential niches of Fe-metabolizing bacteria on Mars. NRFeOM in craters (left), microaerophilic FeOM in the subsurface (middle) and photoferrotrophs in cryoconites (right).

Early Mars likely had niches suitable for acidophilic microorganisms. The identification of Fe minerals such as hematite and jarosite supports the transport of Fe-rich water (Hynek et al., 2002; Örmö et al., 2004) in an acidic environment on the northern hemisphere on early Mars (Klingelhöfer et al., 2004). *Acidithiobacillus ferrooxidans* has been used as a model organism for Martian life under these conditions. This bacterium was isolated from a coal mine drainage in 1951 (Temple & Colmer, 1951) and is an obligate chemolithotroph, capable of Fe(II), elemental sulfur (S^0), sulfide and hydrogen oxidation, as well as reduction of iron(III) and S^0 . Bauermeister et al., (2014) found that it could grow with Martian regolith as the sole nutrient, with a faster growth rate (4-5 days) under oxic than anoxic conditions (8 days) (Quatrini & Johnson, 2018). Biogenic Fe(II) oxidation was shown to be affected by the oxygen concentration and it was lowered when using a Martian O_2 partial pressure (1 Pa). Silva et al., (2024) showed that *A. ferrooxidans* is capable of growing with siderite and vivianite as sole Fe(II) sources coupled to O_2 reduction in oxic conditions and to sulfur (S_8) oxidation and Fe(III) reduction under anoxic conditions. Moreover, it grew with siderite as sole energy (Fe(II)) and C source under oxic conditions, making this a potential prospering metabolism during the transition to an oxidizing Mars, before migration to the subsurface (Silva et al., 2024).

3.2 Life based on Fe on modern Mars

As Martian atmosphere oxidized abiotically (e.g., by O_2) due to the solar wind and radiation about 3.5-3.0 Gyr ago (Cary et al., 2024; Kite et al., 2022; Lanza et al., 2016), it led to a cold dehydrated planet with dangerous UV radiation and a highly oxidizing surface with high concentrations of chlorates and toxic reactive oxygen species (Jakosky et al., 2017). This led to the consideration that under today's environmental conditions, the Martian surface is not habitable. However, oxidizing conditions on Earth have not prevented the proliferation of life.

Antioxidant enzymes - those enzymes used to protect the cells from oxidative stress by neutralizing harmful reactive oxygen species - appeared early in life's history on Earth. Cyanobacteria ancestor ~3.3 to 3.6 billion years ago (Imlay, 2003; Jabłońska & Tawfik, 2021) and archaea ancestors possessed oxidases (Castresana et al., 1994). The environment where life arose on Earth might have had microoxic conditions (Jabłońska & Tawfik, 2021) and oxidases did not appear as a result of the atmospheric oxygenation but might be a casual event potentially triggered by reactive oxygen species and O₂ product of mineral-water interactions (He et al., 2023). Moreover, bacteria have developed mechanisms to avoid or repair damages in the membrane, proteins and/or DNA caused by reactive oxygen species (Addorisio et al., 2022). *Leptospirillum ferrooxidans*, an abundant FeOM in the Tinto River (Spain), induces genes involved in DNA repair and synthesis of carotenoids and cobalamin to respond to oxidative damage (Ferrer et al., 2016). *Leptospirillum ferriphilum* DSM14647 uses the thioredoxin system (composed by thioredoxins Trx and thioredoxin reductase TR) to maintain redox status of proteins under extreme pH conditions (Norambuena et al., 2012). In the case of *Acidithiobacillus ferrooxidans*, another acidophilic FeOM, the damage by reactive oxygen species is avoided by the synthesis of glutathione and manganese superoxide dismutase (Farías et al., 2021). Therefore, oxidizing conditions on the surface might limit the diversity of microorganisms but do not prevent the development of life.

UV radiation is another threat for the development of life in modern Mars. Despite intense UV radiation (Jakosky et al., 2017), photoferrotrophs could still be present in habitable zones within centimeters to meters depth in the ice caps (**Fig. 4**). Dusty water ice could simultaneously provide water and allow enough photosynthetically active radiation to pass through, while attenuating dangerous UV radiation due to the shielding effect of Fe oxide nanophases (Bishop et al., 2006; Khuller et al., 2024). Accounting the use of <1% of the carbon in the sediments of Gale crater, Mars-like conditions have been tested in the laboratory for growing multiple cyanobacteria. *Synechocystis* sp. PCC 6803 grew with Martian regolith simulant amended with nitrate (as sodium nitrate) (Sakon & Burnap, 2006). Antarctic cyanobacteria were also tested and were capable of growing with a Martian regolith simulant. Similar tests employing even stricter Mars-like conditions (e.g. with an analog atmosphere (e.g., Sakon & Burnap, 2006) are encouraged to investigate the potential metabolisms (e.g., photoferrotrophy) that could thrive under those conditions (**Fig. 1**).

A few centimeters below the surface, where UV radiation is lower and Fe(II) is available, microaerophilic FeOM could thrive on Mars (**Fig. 4**). The Martian subsurface could be rich in Fe(II) (50–90% of the total) that is available as an electron donor. The origin of the Fe(II) minerals is mainly basaltic soils (Morris et al., 2006). On Earth, microaerophilic FeOM thrive in environments with low O₂ and high Fe(II) concentrations. However, they are optimal in water-rich environments, such as a water column or at the sediment-water interface (Chan et al., 2016; Emerson et al., 2010). This might be a constraint for their growth on modern Mars where subsurface water is likely limited (Hays et al., 2017). Future work is needed to test the growth of microaerophilic FeOM under Mars-like subsurface conditions.

Fe(III) (oxyhydr)oxides are highly abundant on Mars and thus available as electron acceptor for FeRM. The surface may contain <30 wt% of Fe(III) (Blake et al., 2013; Ramkissoon et al., 2019; Valantinas et al., 2025). The majority is in the form of Fe(III) (oxyhydr)oxides such as Fh, hematite, goethite and magnetite that could be formed by photooxidation (Hurowitz et al., 2017; Nie et al., 2017), weathering (Paul & Mormile, 2020), diagenesis of authigenic Fe(III) (oxyhydr)oxides (e.g., Fh) (Peretyazhko et al., 2018; Rampe et al., 2020), or microbial activity (Hurowitz et al., 2017; Tabata et al., 2021). Furthermore, jarosite, present in the Meridiani Planum (Morris et al. 2004) (and evidence of the presence of water on early Mars) has been proven to be bioavailable for reduction by *Shewanella putrefaciens* (Bingjie et al., 2014; Castro et al., 2013) and by *A. ferrooxidans* (Yang et al., 2020). The latter is also able to catalyze the oxidation of elemental sulfur coupled to Fe(III) reduction under anoxic conditions (Silva et al., 2024).

The recent confirmation of Fh being the most abundant Fe(III) mineral in martian dust (Valantinas et al., 2025) raises a question: why has it not been reduced by FeRB? One possible reason is the limited availability of electron donors, even though the Curiosity rover detected a wide range of organic molecules (Millan et al., 2022). *Geobacter* is capable of using an extensive list of electron donors including alcohols (e.g., propanol and butanol), carboxylates (e.g., acetate, propionate) and H₂ (Nixon et al., 2012) and is a plausible microbial taxon on Mars. However, the available carbon sources on Mars (e.g., amino acids from meteoritic origin) are complex and not bioavailable for oxidation by FeRM enriched from Mars analogs, limiting the feasibility of this microorganisms on Mars, unless simple organics are detected. Moreover, none of the organics found on Mars by these rovers is considered a biosignature and greater efforts are needed in their search and identification (Millan et al., 2021). Another challenge for FeRM are reactive oxygen species. FeRM are more sensitive to these compounds than FeOM (Chen et al., 2022). In fact, FeOM

contribute to the production of reactive oxygen species, hence indirectly affecting the activity of FeRM (Chen et al., 2022). Thus, the activity of FeRM on the Martian surface could be limited by a number of factors.

3.3 Martian BIOS

Given the possibility of Fe-metabolizing microorganisms on Mars, BIOS could have been formed on the early and modern Mars. This offers an opportunity for the search of morphological and molecular Fe-based biosignatures on Mars (Chan et al., 2016), being preserved in the rock record as microfossils, as long as they remain under geochemical conditions that are suitable for preservation (e.g., limited metamorphism) (Hays et al., 2017; Williams et al., 2015). They could also contribute to the extensive Fe(III) (oxyhydr)oxides found on the Mars' surface. For example, mineral-encrusted cells of NReOM could be present in Gale Crater. Twisted stalks and sheaths consistent with microaerophilic FeOM could be present a few centimeters below the Martian surface (see section 3.2). Carbon isotope signatures consistent with phototrophic CO₂ fixation could be associated with Fe(III) minerals within cryoconites (**Fig. 4**). Over time, BIOS could undergo transformation either by FeRM or by diagenetic processes. Hence, our work brings together the repertoire of possible Fe-based biosignatures on Mars.

4. Icy moons

Icy moons refer to satellites orbiting giant planets that are constituted of a water ocean capped by an icy outer layer. Their ocean is a main target for astrobiologists due to their potential habitability. Space missions such as Cassini-Huygens (to Saturn) and Juno (to Jupiter) have provided great insights for the habitability of icy moons. Cassini-Huygens was the first mission to Titan (Saturn's moon) (Lebreton & Matson, 2002; Matson et al., 2002; Sotin et al., 2021), collecting data on its atmosphere and surface. In 2030, the mission Europa Clipper is expected to arrive to Europa and determine whether the subsurface of the moon could harbor life (Becker et al., 2024; Daubar et al., 2024; Pappalardo et al., 2024; Phillips & Pappalardo, 2014; Roberts et al., 2023). In 2031, JUICE mission is planned to explore three of Jupiter's icy moons (Ganymede, Calisto and Europa) (Fletcher et al., 2023; Grasset et al., 2013).

Europa and Enceladus are the most attractive icy moons in terms of habitability. Their ocean is in direct contact with their core, which allows for material exchange and enrichment of the water chemistry (Glein et al., 2015; Porco et al., 2006; S. D. Vance et al., 2018). This is not the case for all icy moons, where some are made of an ice layer between the nucleus and the ocean,

preventing this exchange to happen. Furthermore, Europa and Enceladus present cracks in the outer layer that allow exchanges between the ocean and the surface (Porco et al., 2006). Enceladus stripes are particularly big (extending up to 130 km) with plumes that typically reach hundreds to thousands of kilometers are known to be spewed out (Hansen et al., 2006; Spahn et al., 2006; Villanueva et al., 2023; Waite et al., 2006). The plumes, sampled via Cassini's spectrometers, contain H₂, CH₄, CO₂, NH₃, organic molecules (>200 amu), and phosphate salts (Glein & Waite, 2020; Porco et al., 2006; Postberg et al., 2018, 2023; Waite et al., 2017). Although the presence of these molecules builds on the habitability of Enceladus, up to date no biomarkers (e.g., in the form of lipids) have been identified.

The presence of methane (and the bioessential elements, CHONPS) has led to speculation about the possibility of methanogens and sulfate-reducers in the oceans of Enceladus and other icy moons (Higgins et al., 2024; McCollom, 1999; Taubner et al., 2018; Tenelanda-Osorio et al., 2021; Weber et al., 2023). More recently, Fe-metabolizing bacteria have also gained attention, and Fe has been added to the list of potential active redox elements (Roche et al., 2023; Sahai et al., 2024; Weber et al., 2023; Xu et al., 2025; Zolotov & Shock, 2004b). Here we present some attempts of how to study the habitability of icy moons based on Fe biogeochemistry.

4.1 Enceladus

The saturnian moon, Enceladus, is a small icy moon with a diameter of 500 km. It is the only moon whose interior has been studied *in-situ*. Its plume is the main source of material of Saturn's E ring, where silica nanoparticles were found. These nanoparticles can be formed only under hydrothermal conditions (>90°C), thus suggesting hydrothermalism inside the moon (Waite et al., 2017).

The water-rock interactions in the core offer a habitable place, like it does on Earth's ocean hydrothermal vents (**Fig. 5**). The core of Enceladus is estimated to have a chondritic composition and Fe-bearing hydrated minerals with a porosity 20-30% (Choblet et al., 2017). Models of Enceladus interior suggest that Fe-bearing minerals include hematite, magnetite, siderite, olivine and pyrite (Hamp, 2022; Sekine et al., 2015). As shown above, hematite is an available electron acceptor FeRM (e.g., *Shewanella* species), while siderite can function as electron and carbon source for FeOM (e.g., *A. ferrooxidans*) (Silva et al., 2024). Olivine can also be used as energy source for FeOM (*Pseudomonas* sp. HerB) at temperatures as low as 5°C (Popa et al., 2012). Electron acceptors (NO₃⁻ and O₂) are estimated to be available along with reactive oxygen species

that could be formed at the surface due to the radiation and supplied downward to the ocean through the stripes (Ray et al., 2021). Therefore, Fe(II) oxidation and Fe(III) reduction are both plausible metabolisms on Enceladus.

Figure 5. Potential hydrothermal Fe(II) source on the seafloor of Enceladus and associated Fe microorganisms.

One of the arguments against microbial Fe redox metabolisms is the high pH (9-11) of the ocean. The pH limits the biogeochemical reactions that can take place in a given environment based on thermodynamic constraints (Bethke et al., 2011; Jin & Kirk, 2018) that influence the microbial diversity towards metabolisms favored under those conditions (Frankenberger & Johanson, 1982; Lauber et al., 2009; Leprince & Quiquampoix, 1996). In the case of FeOM, higher pH leads to more rapid abiotic Fe(II) oxidation, such that FeOM can no longer kinetically compete for Fe(II) oxidation. In the case of FeRM, although their optimal pH depends on the mineral reduced (Postma & Jakobsen, 1996), it is typically around 7 (Straub et al., 1998). With Fh, the most thermodynamically favored, the pH upper limit is 8.3 (Jin & Kirk, 2018). At higher pH values (9-12), the energy available for Fe(III) reduction decreases to its lowest point. Then, other metabolisms would be more favorable, such as elemental sulfur and sulfate reduction (Bethke et al., 2011; Flynn et al., 2014).

Geobacter sulfurreducens, a well-known FeRM has been tested under an Enceladan ocean-like conditions and showed growth after 7 days at pH 9 (Roche et al., 2023). Even though higher pH values are expected, *G. sulfurreducens* is not an extremophile, yet it is capable to grow at pH 9. Enceladan polyextreme conditions besides pH (nutrients availability, salinity) could be tested with alkaliphilic FeRM (e.g., *Alkaliphilus metalliredigens* (QYMF) (Roh et al., 2007) (see also Zavarzina et al., 2024). This metabolism would also be favored by the constant supply of H₂, an available electron donor for Fe(III) reduction. Through the plumes, H₂ is expelled at a rate of 1-5×10⁹ mol per year (Waite et al., 2017). This result further supports the need to test other FeRM isolated from alkaline environments (Valencia-Cantero et al., 2007; Williamson et al., 2013).

As a result of the reduction, biogenic minerals are formed, including magnetite, siderite and vivianite (Hansel et al., 2005; Kappler et al., 2021). Considering the capacity for FeRM to thrive in the ocean of Enceladus and taking advantage of the access to the material from the ocean (via plumes), future work should aim to determine the unique properties (e.g., morphology, composition) of the biominerals formed under enceladan conditions that differ from abiotic (e.g., hydrothermal origin) and the detection capabilities of these minerals.

4.2 Europa

Orbiting Jupiter, Europa is one of the most important astrobiological bodies in the solar system. Europa's surface is in constant interaction with Jupiter and to a lesser extent, with Io. Io supplies sulfur species to Europa's surface (through volcanic eruptions and impacts), while Jupiter's magnetosphere bombards Europa with highly energetic protons and electrons. This bombardment triggers photolysis and radiolysis in the surface creating oxidants (e.g., reactive oxygen species, H₂O₂, O₂) that can be transported to the ocean underneath the icy surface. Like Enceladus, it possesses a water ocean below the surface and water vapor plumes have been detected, albeit being about one order of magnitude (~50-200 km) (Roth et al., 2025) smaller than those of Enceladus (~100-9600 km) (Villanueva et al., 2023). These findings have paved the way for the Europa Clipper mission, which is on its way to the moon while this paper is being written.

The possibility of Fe(III) reduction in Europa has been evaluated. With the supply of Fe(II) from the core and the oxidants from the surface, Sahai et al. (2024) proposed an “iron snow” model for the ocean (**Fig. 6**). They propose that the O₂ from the surface would sink (by surface cycling, melting or diffusion (Greenberg, 2010; Pasek & Greenberg, 2012; Vance et al., 2016)) and oxidize the Fe(II) coming from the vents, forming Fe(III) (oxyhydr)oxides that then precipitated to the

seafloor (as “iron snow”, taking inspiration from the “marine snow” phenomena depicting the sinking of organic matter-mineral aggregates in Earth’s oceans). An estimated rate of 2.5×10^{26} cells yr^{-1} of free-living FeRM in the ocean could be expected (Sahai et al., 2024).

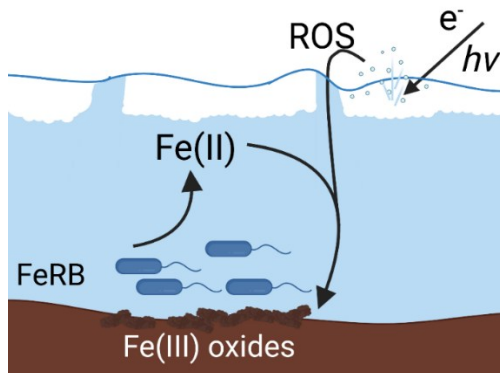


Figure 6. Hypothetic seafloor in Europa. FeRM would reduce the available Fe(III) (oxyhydr)oxides in the seafloor, producing Fe(II) that stays in solution. Fe(II) would be eventually oxidized by the reactive oxygen species produced at the surface and then fall back to the seafloor in the form of abiotic Fe(III) (oxyhydr)oxides.

Contrary to Enceladus, Europa’s ocean is thought to be acidic and its core is thought to be ultramafic. Hydrothermal processes are expected on the seafloor, powered by tidal flexing, leading to interactions between the rocky silicate seafloor and the bottom of the ocean (Hand & Carlson, 2015; S. Vance et al., 2007). Nonetheless, direct confirmation is required to constraint the hydrothermal processes, which are hypothesized to depend on the depth of the mantle (Green et al., 2025). In the plausible scenario of hydrothermal fluxes, the Fe(II) supply from the core and the potential presence of nitrate (from the abiotic oxidation of ammonia) (Weber et al., 2023) can cover the energy requirements of NRFeOM. Moreover, contrary to iron(III) reduction, iron(II) oxidation has higher yields at lower pH values (Peiffer et al., 2024) making Fe(II) oxidation a more suitable metabolism for the seafloor.

4.3 BIOS and biosignatures in icy moons

FeOM offer multiple potential biosignatures that can be targeted on Europa. With a microaerophilic environment (thanks to the O_2 delivered to the ocean from the surface), morphology is one of the best biosignatures one could expect, considering twisted stalks and sheaths that could be produced by microaerophilic FeOM (see section 2.2). However, its detection will be challenging due to the need of sampling and the required techniques. BIOS would be difficult to be found near the surface as they would most likely sink. Microscopy would be needed to describe the

morphologies on the seafloor. Up to date microscopy has been limited to the microscope on board the Phoenix lander on Mars (Hecht et al., 2008) and the microscopic imager on the Mars Exploration Rovers (Herkenhoff et al., 2003). The plumes on Europa might not offer enough material, if the material from the core would ever reach the surface. Then, the mission requires diving in the ocean, which is being proposed (e.g., Konstantinidis et al., 2015; Neveu et al., 2020), but might take a few years to implement due to planetary protection measures, ensuring the safety of both Earth and other planetary bodies for potential cross contamination (National Academies of Sciences, Engineering, and Medicine (U.S.), 2018). Instead, the detection of other lighter molecules could be more promising. For instance, the membrane of the members of *Ferroplasma*, a genus of Acidophilic FeOM, contains caldarchaeetidylglycerol tetraether lipids as a response to the low pH in the environments where they are found. Knowing that cell material can be identified with analog mass spectrometers onboard Europa Clipper (Klenner et al., 2024) this specific lipid could be a targeted molecule to search in Europa's ocean (e.g., via mass spectrometry) (Golyshina & Timmis, 2005).

On Enceladus, FeRM would be more favorable than FeOM given the high pH. The discovery of alkaliphilic FeRM (Switzer Blum et al., 1998) offers an opportunity to test the habitability of the moon and the biosignatures to look for. Alkaliphilic FeRM withstand pH values of 9.0 to 10.4 (Gorlenko et al., 2004; Ye et al., 2004) and a representative species of *Bacillus* (strain SFB) pushes the limit to 11.0 (Pollock et al., 2007). At this pH values, Fe(III) reduction is challenging and more thermodynamically favored in the presence of electron shuttles (Wang et al., 2018). Siderite, magnetite and vivianite are within the main Fe(II)-bearing minerals that result from microbial Fe(III) reduction. The identity of the reduction products highly depends on the mineralogy of the initial Fe(III) (oxyhydr)oxides and the presence of phosphate and volatiles as well as OC identity and concentration (Fredrickson et al., 1998; O'Loughlin et al., 2019, 2021; Roh et al., 2003). With increasing concentrations of phosphate, the reduction rate becomes slower but yields higher reduction extents and favors the production of vivianite and green rust while magnetite is formed at lower concentrations (O'Loughlin et al., 2021). The presence of N₂ also promotes magnetite while CO₂ promotes siderite formation (Roh et al., 2007). On Enceladus, the recent discovery of phosphate (Postberg et al., 2023), the presence of H₂ (Waite et al., 2017) and the carbonate-rich ocean (Glein & Waite, 2020) set constraints on the Fe(II)-bearing mineral products of possible microbial Fe(III) reduction, most likely in the form of siderite. In the presence of OC, siderite forms in spindle-, rod-, peanut-, and dumbbell-like structures, which is not the case in the OC-free abiotic formation (Han et al., 2024). Considering the predicted presence of siderite in the

Enceladus core (Hamp, 2022), once again microscopic techniques should once again be considered for determining the biogenicity of these Fe biominerals.

5. Lessons from model habitats on earth

5.1 Mars

Modern model habitats on Earth are great field sites for understanding life and their signatures under similar conditions on Mars. One model habitat is the Río Tinto (Spain) as an early Mars analog. Located in the Iberian Pyrite Belt, Río Tinto is a river with high concentration of metals, pH 2.3 and high concentration of Fe(III) (Amils et al 2014) comparable to the Noachian era (4.1 to 3.7) of Mars. Its similarity to Meridiani Planum is striking given the abundance of Fe(III) (oxyhydr)oxide (goethite) and sulfate (jarosite) minerals that are formed at a highly acidic pH (Finkel et al., 2023). These minerals are formed in close association with microorganisms in Río Tinto today, leaving behind deposits such as iron terraces and microbialites. Proving that these deposits can only be formed in the presence of life is a huge endeavor that is being continued today. Fe(III) minerals also favor the preservation of organic biosignatures, especially in the form of lipids (Fernández-Remolar et al., 2021; Lalonde et al., 2012). While the Río Tinto is an excellent model habitat that reproduces biogeochemical Fe cycling in an acidic environment, it has one huge discrepancy compared to Mars in terms of temperature. Temperature on Mars is often below zero, which means that psychrophiles (organisms capable of living with temperatures below 20°C) are the best model organisms instead of mesophiles (whose optimal growing temperature is within 20-45°C).

Hence, other model Martian habitats are focused on subglacial terrestrial environments such as the Atacama Desert, Antarctica and ice caps (Fisher & Schulze-Makuch, 2013). The Atacama Desert is a well-known Mars analog for the study of the Amazonian period (2.9 Gyr ago to present). It has the highest UV radiation levels on Earth and oxidizing soils due to the presence of perchlorates, with similar concentrations to Martian surface (Azua-Bustos et al., 2020; Hecht et al., 2009). Within the desert, two sites are recognized as the most Martian-like ecosystems: the coastal range and the hyperarid core (Azua-Bustos et al., 2022; Navarro-González et al., 2003). Despite the apparent hostile conditions of the desert, microorganisms have been identified, challenging the general view of oxidizing Mars as inhabitable and giving clues of which biosignatures to look for (Azua-Bustos et al., 2023; Bartholomäus et al., 2024).

The ice caps are also analogs of modern Mars, particularly, the cryoconites. Cryoconites are holes in the ice caps, formed by the condensation of dust particles that fall in the surface and favor melting of their surroundings. They host complex communities that include microorganisms and even small invertebrates (e.g., tardigrade), dominated by cyanobacteria (Cameron et al., 2016; Mueller 2001). Photosynthetic microorganisms could thrive in Martian cryoconites that could occur in its ice caps (Clow, 1987) as demonstrated by Khuller et al., (2024). Although non-photosynthetic Fe-utilizers have been enriched from similar cold environments (Nixon et al., 2017), no other study has yet truly focused on analyzing biosignatures under those conditions based on an Fe-based perspective.

5.2 Icy moons

Analog studies for icy moons are more recent, compared with the number of studies and analog sites of Mars. Research has been focused on laboratory ice analogs to study the effect of radiation on the surface of the moons and constraining the ability of detection of the organic material coming out of the plumes (Khawaja et al., 2024; Klenner et al., 2020, 2024; Robinson et al., 2023). Recently, several sites in the Arctic and Antarctica have gained attention for the search of extremophiles and for testing life-detection instruments. Subsurface lakes in the Arctic and Antarctica resemble subsurface oceans in icy worlds (Cassaro et al., 2021). Despite being 4 km beneath the ice sheet (Siebert et al., 2001), lake Vostok in Antarctica host a community of microbes and enough dissolved OC (~0.5 mg/l) to sustain heterotrophic organisms (Priscu et al., 1999). In Ellesmere Island in the Canadian High Arctic, the supraglacial spring system of Borup Fiord Pass is an analog of the European surface. There, the ice contains sulfur minerals with ongoing sulfur chemistry that mimic those thought to be happening on the surface of Europa (Grasby et al., 2003). Also in the Arctic, on the West Svalbard continental slope, the gases trapped in the ice cap have been used as analog of the ice sheet and plume composition of Enceladus, in an attempt to investigate a potential methane cycle (Franchi et al., 2025).

With respect to the seafloor, hydrothermal systems are analogs to the expected water-rock interactions in the interiors of icy moons. One example of a hydrothermal system on land can be found at Deception Island, Antarctica. The microorganisms there survive across extreme temperature gradients, from the hot fumaroles (100°C) to the subzero temperatures in the glacier (Bendia et al., 2018; Lezcano et al., 2019; Uribe-Redlich et al., 2024). Oceanic hydrothermal vents are also good analogs of the icy moons' seafloor, characterized by high pressures and temperature gradients. Lost City in the mid-Atlantic ocean is widely studied due to its rich diversity of organisms

despite the extreme conditions of high pressure, temperature and limited nutrients. Its ultramafic nature, the high concentration of H₂ and abiotic production of organics make this and other hydrothermal vents (e.g., Mid-Cayman Rise) the best seafloor hydrothermal vent analogs (Kelley et al., 2005; McDermott et al., 2015).

6. Future perspectives

Fe is ubiquitous in life and on multiple bodies in the solar system. Here, we have highlighted several examples of Fe-metabolizing microorganisms on Earth and the biosignatures that they may leave behind. Our review provides new perspectives on the search for Fe-based biosignatures on Mars and the icy moons.

First, the preservation of BIOS should be investigated in more detail under Martian subsurface or icy moon-like conditions. This area can build upon existing studies that investigated BIOS and/or Fe(III) (oxyhydr)oxide preservation under Earth-like diagenetic conditions (up to 250°C/140 MPa) (Orange et al., 2014; Picard et al., 2015; Westall et al., 2015) or in Mars analog sites (Iron Mountain, CA; Yilgarn Craton, Western Australia; Gunnuhver, Iceland; Hveravellir, Iceland; the Río Tinto and Atacama Desert) (Azua-Bustos et al., 2022; Fernández-Remolar et al., 2021; Williams et al., 2021).

Second, new techniques are needed onboard rovers to better target strategic biosignatures. For example, microscopic techniques (ideally with resolution to the nanoscale) are needed to be able to identify BIOS-like morphology (e.g., sheaths, stalks). The inclusion of scanning electron microscopy, capable of resolving cell-like structures and to correlate morphology with mineralogy and organic content, will be particularly helpful. Furthermore, future work should also include isotopic analysis of organic matter associated with Fe minerals, as C and N could have distinct biological isotopic fractionation (Fifer & Wong, 2024). However, the implementation of these techniques still requires extensive research on miniaturization. This supports the need of sample return missions to incorporate techniques that are still too challenging to include on board space missions (Kminek et al., 2022; McCubbin et al., 2025; Muirhead et al., 2020; Tait et al., 2022).

Finally, detection of Fe-based biosignatures should also take into account capabilities of ongoing and upcoming mission to Mars and icy moons. The Perseverance rover on Mars - equipped with Raman and X-ray spectrometers - (Von Ehrenfried, 2022), have continued to identify Fe minerals (vivianite, iron oxides enriched in Cr and Ti, greigite) that support inferences for habitability

(Hurowitz et al., 2025; Kizovski et al., 2025; Mansbach et al., 2024). Upcoming missions such as Rosalin Franklin rover, Europa Clipper and JUICE will provide intriguing clues on the habitability of Mars and Europa. The Rosalind Franklin rover has a Raman spectrometer (RLS, 532 nm) onboard that will continue the characterization of the mineralogy and the possibility of detecting organic compounds (Lopez-Reyes et al., 2014; Rull et al., 2017; Veneranda et al., 2020). For studying Europa, Europa Clipper carries the mapping imaging spectrometer for Europa (MISE) that includes an infrared spectrometer capable of detecting absorption features of iron-bearing minerals (oxides, hydroxides, sulfates) (Blaney et al., 2024). It also carries the surface dust analyzer (SUDA), a mass spectrometer that can capture iron containing particles (Kempf et al., 2025), and a magnetometer that could detect magnetic anomalies caused by iron minerals (Kivelson et al., 2023). Hence, the development of proxies (i.e., indirect measurements) that can be applied remotely is essential in the search of these biosignatures in remote places (e.g., icy moons) where *in situ* measurements are not possible.

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Glossary

Assimilatory metabolism: microbial process where inorganic compounds are used to generate biomass.

Banded iron formations (BIF): sedimentary rocks composed of alternating layers of iron minerals and silica.

Dissimilatory metabolisms: microbial process where inorganic compounds are used to generate energy.

Electron acceptor: molecule, atom or ion that receives the electrons during cellular respiration.

Electron donor: molecule, atom or ion that provides the electrons during cellular respiration.

Extracellular polymeric substances (EPS): mixture of high-molecular-weight biopolymers (e.g., polysaccharides, proteins, nucleic acids), secreted by microorganisms for stability (mechanical or chemical) and protection.

Fe(II)-oxidizing microorganism (FeOM): organisms that oxidize Fe(II) to Fe(III) to obtain energy for growth.

Fe(III)-reducing microorganism (FeRM): organisms that respire Fe(III) as the final electron acceptor, reducing it to Fe(II).

Mesophile: organisms whose optimal temperature for growth and reproduction is typically between 20-45°C.

Niche: environmental conditions that a species requires to grow and reproduce.

Nitrate-reducing Fe(II)-oxidizing microorganisms (NRFeOM): Fe(II)-oxidizing microorganisms that couple Fe(II) oxidation to nitrate reduction to gain energy.

Photoferrotroph: organisms that oxidize Fe(II) using light energy to fix carbon dioxide.

Psychrophile: organisms whose optimal temperature for growth and reproduction is typically between 20-45°C.

Reactive oxygen species (ROS): highly reactive molecules derived from molecular oxygen (O₂) including free radicals and non-radical oxygen-containing compounds (e.g., ·OH, O₂^{·-}, H₂O₂).

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Chapter 3. Authors contribution

Bioavailability of biogenic iron(III) oxyhydroxides for Fe(III)-reducing bacteria

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This study was conceptualized by M. Mansor and A. Kappler. M. Mansor and A. Kappler acquired the funding. M. Mansor, A. Kappler and I designed the experiments. I performed the experiments. Tsz (Alex) Ho Chiu carried out the XRD and BET measurements. I analyzed the data with help of M. Mansor and A. Kappler. I wrote the original draft of the manuscript and all authors contributed to the review and editing of the final manuscript.

Chapter 3. Bioavailability of biogenic iron(III) oxyhydroxides for Fe(III)-reducing bacteria

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Abstract

Iron-metabolizing bacteria play a key role in the biogeochemical Fe cycle, linking it to the bioavailability and transformation of other bioessential elements such as carbon, nitrogen and phosphorus. Fe(III)-reducing bacteria use Fe(III) as an electron acceptor and Fe(II)-oxidizing bacteria oxidize Fe(II) to gain energy, forming biogenic iron(III) oxyhydroxides (BIOS). Little is known about the bioavailability of the Fe(III) associated to BIOS (BIOS_{Fe(III)}), limiting predictions of its role in the mobility of other bio-essential elements. Here we tested the bioavailability of BIOS_{Fe(III)} as a function of (a) BIOS origin (phototrophic and mixotrophic/autotrophic nitrate-reduction coupled to Fe(II) oxidation), resulting in a gradient of cell-mineral associations from encrustation to loose attachments, (b) two model Fe(III)-reducing microorganisms: *Shewanella oneidensis* MR-1 and *Geobacter sulfurreducens* PCA, (c) presence or absence of added organic carbon (lactate or acetate), and (d) before and after freeze-thawing, simulating conditions relevant to temperate environments. Statistical redundancy analysis showed that despite variations in the properties of the BIOS (e.g. particle size, surface areas, organic content) due to its origin and freeze-thaw treatment, organic carbon availability was the main factor controlling BIOS_{Fe(III)} reduction rates and extents. With added organic carbon, BIOS_{Fe(III)} were reduced completely, with faster rates observed for *G. sulfurreducens*, suggesting limited availability of BIOS-associated organic carbon (BIOS_{OC}) to these microorganisms. BIOS_{Fe(III)} was also more bioavailable than synthetic 2-line

ferrihydrite, attributed to increased redox reactivity originating from BIOS_{OC} (e.g., residual cells and extracellular polymeric substances). Freeze-thawing, a process relevant in high-latitude zones, mountain glaciers, and other temperate climate regions across Earth, mostly decreased the rate and extent of BIOS_{Fe(III)} reduction, although the magnitude and direction of the effect depend on the specific variable combination. Thus, we conclude that environmental organic carbon availability is the primary factor controlling the bioavailability of BIOS_{Fe(III)}, with subsequent effects on the transformation of other bioessential elements.

Introduction

Biogenic iron(III) oxyhydroxides (BIOS) are mixtures of Fe(III) nanominerals, cells and extracellular polymeric substances (EPS) produced by Fe(II)-oxidizing bacteria (FeOB). The mineral component typically consists of ferrihydrite ((Fe^{III})₄₋₅(OH,O)₁₂, Fh), goethite (α -Fe^{III}O(OH)) and amorphous Fe(III) phosphates depending on the geochemical conditions of the environment or the experimental conditions (Abramov et al., 2022; Angelova et al., 2015; Ferris, 2005; Gault et al. 2012; Kikuchi et al. 2014; Miot et al. 2015, 2009; Nedkov et al., 2016). The organic part is mostly lipid and polysaccharide moieties associated with aliphatic groups in the outer part of the minerals, while carboxylic and aromatic moieties are incorporated within the structure (Chan et al., 2009; Sowers et al., 2019; Whitaker et al., 2021). At circumneutral pH and microoxic conditions, biotic Fe(II) oxidation occurs at a higher rate than abiotic oxidation (Baker et al., 2023; Dopffel et al., 2022; Druschel et al. 2008). Furthermore, there are numerous observations of orange-colored microbial mats rich in Fe(III) nanominerals across different environments (Brooks and Field 2020; Garber et al. 2021). Hence, BIOS represent a significant component of Fe(III) oxyhydroxides minerals in environments where FeOB are present.

The concept of a “rusty carbon sink” has been greatly explored in recent years, underscoring the importance of Fe(III) (oxyhydr)oxides in controlling the bioavailability of organic carbon (Goebel et al., 2005; Yudina & Kuzyakov, 2019). On average, about 40% and 19% of the total OC (TOC) is associated with Fe in terrestrial soils and marine sediments, respectively (Jia et al., 2024; Lalonde et al., 2012, Helffrich & Wood, 2001; McDonough & Sun, 1995). These Fe(III) minerals can be altered by Fe(III)-reducing bacteria (FeRB) that reduce Fe(III) to Fe(II) coupled to the oxidation of electron donors such as OC, CH₄, H₂, or sulfur species (Chen et al. 2025; Kappler et al., 2021; Knoll, 2012; Konhauser et al., 2011). The extent of Fe(III) reduction is governed by the crystallinity and size of the minerals and the availability of biodegradable OC (Roden, 2003, 2004; Xu et al., 2025). Intriguingly, the Fe(III) associated to BIOS (BIOS_{Fe(III)}) has been shown to be

more bioavailable for microbial reduction than that in synthetic Fe(III) (oxyhydr)oxides (Ferris, 2005; Langley, et al., 2009; Straub et al. 1998). The increased reactivity is attributed to the lower crystallinity and higher surface area of BIOS compared to synthetic Fe(III) (oxyhydr)oxides (Chan et al., 2009; Miot et al., 2015; Toner et al., 2009; Whitaker et al., 2021). Specifically, Langley et al. (2009) found that *Shewanella putrefaciens* CN32 reduced BIOS twice faster compared to synthetic two-line ferrihydrite (Langley et al., 2009). Moreover, BIOS of different origins exhibit different reactivity— BIOS_{Fe(III)} from *Leptothrix* spp were reduced twice faster than those from field sites dominated by *Gallionella* spp (Langley et al. 2009; Najem et al. 2016). These studies raised the question as to whether other BIOS will also display different bioavailability depending on their origin, and why (*i.e.*, due to variable crystallinity and organic association). Furthermore, as diverse FeRB species exist in nature, there is the question of whether different BIOS might exhibit varying reactivity to different FeRB.

The “rusty carbon sink” concept is especially relevant to environments undergoing rapid thawing, such as in high-latitude zones and mountain glaciers. As ice thaws due to climate change, previously buried organic carbon is suddenly at risk of being decomposed into greenhouse gases such as CO₂ and CH₄. Association of organic carbon with Fe(III) (oxyhydr)oxides typically results in protection of the organic carbon from microbial degradation (Adhikari et al., 2019). Decomposition rate of glucose, for instance, is >99.5% faster for free glucose in soil than in glucose adsorbed on synthetic ferrihydrite (Porras et al., 2018). Nonetheless, the reductive dissolution of Fe(III) (oxyhydr)oxides by FeRB releases Fe-associated organic carbon that can be further metabolized to simple organic carbon (e.g., acetate, lactate), CO₂ and CH₄ (Adhikari et al., 2017; Chung et al., 2023; Tian et al., 2025). This association is also observed in biofilms, where FeRB typically reside close to or within biofilms of FeOB, together contributing to a biological Fe cycle on small spatial scales (Brooks & Field, 2020; Garber et al., 2021). At the moment, there is limited knowledge on whether BIOS-associated organic carbon (BIOS_{OC}) can be degraded or released by FeRB.

In high latitudes and mountain glaciers, as well as in temperate climate regions across Earth, soils undergo regular freeze-thaw cycles that affect Fe-organic carbon associations. On the one hand, freeze-thawing is known to facilitate aggregation (Wu et al., 2022) and to slow down mineral transformation due to the lower temperature (Mullin 2004; Putnis 2009). On the other hand, freeze-thawing can also accelerate mineral transformation by generating high surface area fractures (Patzner et al. 2020) or promoting localized high ion concentrations (e.g., O₂, H⁺, Fe²⁺) in microenvironments around the minerals (Patzner et al., 2022; Zhai et al., 2021). Freeze-thawing

can also promote the release of organic carbon from mineral aggregates (Wu et al., 2022) or promote the degradation of complex organic carbon (Fellman et al., 2008; Pokrovsky et al., 2018; Payandi-Rolland et al., 2021), potentially increasing its bioavailability. Hence, freezing causes a variety of interrelated effects in soils, necessitating laboratory experiments to quantify its net effects on the bioavailability of Fe(III). BIOS are also exposed to freeze-thawing. Thus, controlled laboratory experiments using freeze-thawed BIOS are necessary to directly quantify the net Fe(III) reduction rate and extent, as well as providing mechanistic insights into how changes in properties relate to changes in bioavailability.

In this study, we investigated the bioavailability of BIOS as a function of multiple variables: three origins of BIOS, two FeRB, with and without added organic carbon and before and after freeze-thawing. This combination of variables resulted in a total of 24 setups, allowing us to investigate the bioavailability of BIOS with wide implications across diverse environmental systems. Under some conditions, we also compared the bioavailability of BIOS to synthetic ferrihydrite, underlining the importance of performing experiments with naturally relevant materials to avoid interpretation based solely on pure lab-synthesized minerals with less relevance to the environment.

Methods

Fe(III) mineral sources

All glassware used for cultures was washed in acid (1 M HCl), then rinsed three times with double deionized water (DDI, Millipore, $>18 \text{ M}\Omega \text{ cm}^{-1}$), and subsequently baked at 180°C for 4 h and 30 min prior to use. Sampling was conducted under aseptic and anoxic conditions in a glovebox (MBraun Unilab Workstation, 100% N_2 atmosphere).

BIOS were obtained from three Fe(II)-oxidizing bacteria strains with different Fe(II) oxidation mechanisms: photoferrotrophy by *Rhodopseudomonas palustris* TIE-1 (hereafter TIE-1), chemototrophy coupled to nitrate reduction by enrichment culture KS (hereafter KS), and mixotrophy coupled to nitrate reduction by *Acidovorax* sp. BoFeN1 (hereafter BoFeN1) (**Text S1**). The FeOB were grown in 22 mM bicarbonate-buffered mineral medium at pH 7 (Ehrenreich & Widdel, 1994) in 100 mL serum bottles with 50 mL headspace of N_2/CO_2 (90:10 v/v) in the dark at 28°C for BoFeN1 and KS, and at 20°C under illumination for TIE-1. The BoFeN1 medium had a lower phosphate concentration (1 mM) in addition to 3.5 mM NaCl to maintain the ionic strength in the medium. It was then amended with 10 mM Fe(II) (as Fe_2Cl), 6 mM NO_3^- (as NaNO_3) and 2 mM acetate (as CH_3COONa). KS medium was amended with 10 mM Fe(II) and 2 mM NO_3^- . TIE-1

medium was amended with 3 mM Fe(II), *i.e.* a lower initial Fe(II) concentration than the other strains, because of its faster metabolism at this concentration. We acknowledge that at lower Fe(II) concentrations TIE-1 BIOS are richer in ferrihydrite, while at higher concentrations and therefore longer incubation time, TIE-1 BIOS have a higher proportion of crystalline minerals due to recrystallization and reaction with the Fe(II) in solution (Jiao et al. 2005; Nikeleit et al. 2024). The media were inoculated with 1% (v/v) of a preculture in the case of KS, 5% (v/v) of BoFeN1 and 3% (v/v) of TIE-1. Precultures were grown in the same conditions as described above.

For native BIOS experiments, BIOS were collected when Fe(II)/Fe(total) ratio was $5.7 \pm 1.5\%$, $8.0 \pm 2.0\%$ and $7.0 \pm 0.2\%$ for TIE-1, BoFeN1 and KS, respectively, indicating near complete oxidation. For experiments with freeze-thawed BIOS, the initial Fe(II)/Fe(total) ratio for BIOS of TIE-1, BoFeN1 and KS was $6.9 \pm 0.6\%$, $13.4 \pm 0.3\%$ and $27.1 \pm 0.5\%$, respectively. This corresponded to 6 days of incubation for BoFeN1 and KS, and 12 days for TIE-1. To collect the BIOS, the serum bottles with the cultures were centrifuged at 12,045 g for 2 minutes and the supernatant was exchanged with fresh FeRB medium (composition detailed below). This was repeated three times for each bottle. After the last centrifugation, the supernatant was removed, and all the BIOS were finally collected, resuspended with FeRB medium, and stored anoxically and aseptically at 4°C. For freeze-thawed BIOS setups, the 200 mL serum bottles containing BIOS (after washing) were left to freeze in a freezer at -20°C for 13 days. After this time, the bottles were left to thaw at room temperature before transferring the BIOS for the Fe(III) reduction experiments with FeRB, keeping the anoxic and aseptic conditions.

2-line ferrihydrite was synthesized according to Schwertmann & Cornell (2000). 40 g of $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ were dissolved in 500 mL MilliQ water and the pH of the solution was adjusted to 7.3 by titration with 1 M KOH. After 2 hours of incubation, the pH of the solution was adjusted to 7.5 with KOH. The minerals were washed 4 times with MilliQ water before resuspension in water to a concentration of ~335 mM Fe total.

Cultivation of FeRB and experimental setup

Fe(III)-reducing bacteria have developed multiple mechanisms to reduce Fe(III), including direct contact between minerals and the cell membrane, conductive pili, electron shuttles and chelation. Here we tested BIOS bioavailability for FeRB *Shewanella oneidensis* MR-1 (hereafter *S. oneidensis*) and *Geobacter sulfurreducens* PCA (hereafter *G. sulfurreducens*) as model organisms, whose primary reduction mechanisms differ (electron shuttles and direct cell-mineral contact,

respectively; **Text S2**). The FeRB were grown in 30 mM bicarbonate-buffered mineral medium at pH 7 (Ehrenreich & Widdel, 1994) in 100 mL serum bottles with 50 mL headspace of N₂/CO₂ (90:10 v/v %) in the dark at 28°C. Six bottles were amended with ~6 mM of BIOS_{Fe(III)} from each of the three FeOB (18 bottles in total). Of these, 9 bottles were amended with ~4 mM of an additional carbon source: acetate for *G. sulfurreducens* and lactate for *S. oneidensis*. The same setup applies for experiments with freeze-thawed BIOS. The initial cell concentration in the experiment was ~5 x 10⁵ cells/ml, achieved by inoculating the media with 5% (v/v) of a preculture containing ~10⁷ cells/mL of each FeRB strain. Cell concentration was calculated using optical density (OD) and calibration curves from our laboratory in the case of *S. oneidensis* (OD = 0.095 ± 0.002) and from Kubannek et al. (2020) in the case of *G. sulfurreducens* (OD = 0.17 ± 0.04). Precultures were grown using fumarate as an electron acceptor. Duplicates of FeRB cultures with ~5 mM acetate/lactate as an electron donor and ~6 mM ferrihydrite as an electron acceptor were set up to check for cell viability in some cases. Negative controls for both setups (with and without carbon) contained ~6 mM of Fe(III) of each BIOS and acetate/lactate as stated above.

Geochemistry

Every two days, 4 mL of sample were taken from each replicate for the first 12 days with a syringe under anoxic conditions in a glovebox. Due to the lower Fe(III) reduction rate of *S. oneidensis*, sampling was done every 4-7 days after day 12. Samples were split into two vials, one for centrifugation at 12,045 g for 5 min and the other one (without centrifugation) for Fe(II) and Fe(total) content (150 µl). From the supernatant of the centrifuged samples, aliquots were taken for dissolved organic carbon (in the form of non-purgeable organic carbon) and Fe²⁺ content. Samples for Fe measurements were diluted 1:1 with 1 M HCl inside the glovebox and further diluted with 1 M HCl prior to analysis.

Fe content (Fe²⁺, Fe(II), and Fe total) was quantified in triplicate by colorimetry using the ferrozine assay (Hegler et al., 2008; Stookey, 1970). For quantification of Fe(II) and Fe²⁺, 20 µl of sample were added to 80 µl of 1 M HCl in a 96-well plate followed by 100 µl of ferrozine reagent. After 5 min, the ferrozine-Fe(II) complex was measured at 562 nm in a plate reader (Multiskan GO; Thermo Fisher Scientific, USA). For Fe total (Fe(tot)), 20 µl of sample were added to 80 µl of hydroxylamine hydrochloride (10% w/v in 1 M HCl) in a 96-well plate. After 30 min of reaction, the ferrozine reagent was added, and the ferrozine-Fe(II) complex was measured after 5 min of incubation. All samples were measured in triplicate and in the dark. Each 96-well plate also contained triplicates of 50, 100, 200, 400, 600, 800, and 1000 µM Fe(II) ethylenediammonium sulfate

tetrahydrate for standard curve calibration. The aliquot for dissolved organic carbon concentration was diluted 1:4 in 80 mM HCl and measured in triplicates with a TOC analyzer (multi N/C 2100S, Analytik Jena AG, Germany). Each run contained standards of 5, 10, 20 and 40 mg/L OC for calibration.

Solid phase analysis

Four aliquots of 2-5 mL each from the washed BIOS and from the freeze-thawed BIOS were centrifuged, and the supernatant was discarded. The solids were then dried (>24 h) and stored under anoxic conditions until total carbon analysis, surface area analysis, μ -X-ray diffraction (μ -XRD), and Mössbauer spectroscopy.

The total organic carbon content in the solid phase was determined in duplicates with a soliTOC (Cube, Elementar, Germany). The specific surface area (SSA) was determined with the Brunauer-Emmett-Teller equation (BET) (Brunauer et al., 1938) by adsorption/desorption of N₂ using a Micromeritics Gemini VII surface area and porosity analyzer (Micromeritics Instrument Corporation, USA). At partial pressures ranging from 0.05 to 0.3 P/P₀ and at 77 and 298 K, N₂ adsorption was quantified from outgassed samples over a 24-hour period.

μ -XRD was performed with a Bruker D8 Discover GADDS XRD2 micro-diffractometer equipped with a standard sealed tube with a Co-anode (CoK α radiation, λ = 0.17903 nm, 30 kV/30 mA). The measurement time was 240 s at detector positions 15° and 40°. Resulting diffractograms were analyzed using the software Match! (version 3.6.2.121). Crystallite size (D) was calculated using the Scherrer equation (Patterson, 1939):

$$D = \frac{k\lambda}{B} \cos(\theta) \quad (1)$$

where k is the Scherrer constant (assumed to be 0.9), λ is the X-ray wavelength (0.1789 nm for CoK α) and B is the full-width half-maximum (FWHM) of the diffraction peak.

The identification of Fe phases within the BIOS was performed by Mössbauer spectroscopy. The dried BIOS were homogenized by mortar and pestle in the glovebox before loading them into Plexiglas holders (area: 1 cm²), forming a thin disc. The holders were frozen and stored anoxically until analysis. Holders were inserted into a closed-cycle exchange gas cryostat (Janis cryogenics) under a backflow of He gas to minimize exposure to air. Spectra were collected at 77 K and 30 K using a constant acceleration drive system (WissEL) in transmission mode with a ⁵⁷Co/Rh source. All spectra were calibrated against a 7 μ m thick α -⁵⁷Fe foil that was measured at room

temperature. Analysis was carried out using Recoil (University of Ottawa) and the extended Voigt Based Fitting (VBF) routine (Lagarec and Rancourt, 1997). The half width at half maximum (HWHM) was constrained to 0.122 mm/s during the fitting process.

Size distribution analysis

Particle size was measured for the original and freeze-thawed BIOS. The unfiltered particulate fraction was measured using a particle size analyzer (Mastersizer 2000, Malvern Instruments, United Kingdom) with distilled water as dispersant, adding the samples directly from the serum bottles with the cultures, one mL at a time with a pipette until the obscuration was between 10-20% in the Hydro 2000S dispersion unit (total volume of 150 mL). A total of 10, 18 and 4 mL were added for the original BIOS of BoFeN1, TIE-1 and KS, respectively. For the freeze-thawed BIOS, 12, 15 and 8 mL were added for BoFeN1, TIE-1 and KS, respectively. Measurements were conducted four times per sample, on duplicate samples, with three cleaning cycles in between each sample.

The particle size and zeta potential of the colloidal fraction (operationally defined as $<1.2\ \mu\text{m}$ based on the filter size) was measured by Dynamic Light Scattering (DLS) using a Zetasizer Nano (Malvern Instruments) with three runs of 20 s each. Samples were collected in a glovebox by passing an aliquot of 2 mL through a $1.2\ \mu\text{m}$ pore size filter (Acrodisc 32 mm syringe filter) and stored there until measurement. Then, 0.9 mL of the filtered samples were placed in a DTS1070 disposable folded capillary cell for both size (3 runs, 20 s each) and surface charge (3 runs, 50 scans each) analysis. The attenuation and voltage parameters were determined automatically by the instrument. The measured electrophoretic mobility was converted to surface charge using the Smoluchowski method (Hunter 1981).

Scanning Electron Microscopy

At the end of the incubation of FeOB, BIOS were collected and imaged with a Scanning Electron Microscope (SEM). An aliquot of 200 μL of sample were fixed in 2.5 % (aq) electron microscopy-grade glutaraldehyde and sequentially dehydrated with 25%, 50% 75%, and 100% (v/v) ethanol and finally 100% (v/v) hexamethyldisilazane (HMDS) on a round glass coverslip previously coated with a 0.01% w/v solution of Poly-L-Lysine to provide a hydrophilic surface for attachment. The samples on the glass slide were dried overnight and attached to aluminium stubs using carbon adhesive tabs. This was then sputter-coated with a 10 nm gold layer (sputtering 50 s at 5×10^{-2} mbar, 30 mA, and a working distance of 35 mm) using a BAL-TEC SCD 005 sputter coater.

Imaging was performed with a ZEISS Crossbeam 550/5502 L, with an acceleration voltage of 5 kV, and the SESI (secondary electrons - secondary ions) detector.

Statistical analysis

ANOVA analysis to test significant differences between treatments were performed in Origin 2025 (OriginLab Co., USA). The Fe(III) reduction extents reported here and used for calculations refer to the Fe(II)/Fe(total) ratios that were not subtracted with the ratios at timepoint 0. The Fe(III) reduction rates were calculated from the linear phase of the reduction rate, excluding the lag phase (which could cause potential biases from initial inoculation) and the stationary phase when Fe(III) reduction had stopped. The Redundancy analysis (RDA) was performed in CANOCO 5 software (Microcomputer Power Co., USA).

Results

BIOS characterization before and after freeze-thawing

Mineralogy and cell-mineral associations

In this study, we subjected original and freeze-thawed BIOS from three different FeOB with different metabolisms to reduction by FeRB. BIOS were first characterized through a suite of analytical techniques before the reduction experiments. All BIOS consisted of a mixture of different Fe(III) oxyhydroxides with varying crystallinities. Micro-XRD indicated that goethite was the primary crystalline phase in all FeOB (**Fig. S1**). Vivianite was also present in KS, but its origin is abiotic, due to the precipitation of Fe(II) and phosphate initially present in the medium (4 mM). Mössbauer spectroscopy showed that KS BIOS had the most complex composition with more than 50% of the minerals as ferrihydrite. In BoFeN1 and TIE-1 BIOS, poorly crystalline minerals such as ferrihydrite and lepidocrocite were together present in higher proportion than that of crystalline goethite (**Figs S2, S3; Text S3; Table S1**). SEM images showed an increasing degree of cell encrustation with minerals in the order of TIE-1 < KS < BoFeN1 (**Fig. S1**). The mineralogical assemblages and cell-mineral associations are broadly consistent with what has been observed in past studies (**Text S1 and S4**). Freeze-thawing led to no changes in mineralogy or XRD-derived crystallite size (**Table 1**) and goethite remained the primary crystal phase in the system, in addition to vivianite in the case of KS (**Fig. S1**).

Table 1. Crystallite size (nm) of TIE-1 as determined from the Scherrer equation (**Eq. 1**) based on XRD reflection patterns. Grey values indicate lower values when comparing original vs freeze-thawed TIE-1.

Mineral phase	2- θ (°)	Replicate	TIE-1-1		BoFeN1		KS	
			Original	Freeze-thawed	Original	Freeze-thawed	Original	Freeze-thawed
Goethite	25	a	9.58	13.32	10.73	10.88	-	-
		b	-		9.67			
Goethite	63	a	11.39	9.30	15.51	19.05	29.34	12.34
		b	8.90		14.86		17.02	
Vivianite	15	a					12.98	16.16
		b					14.96	

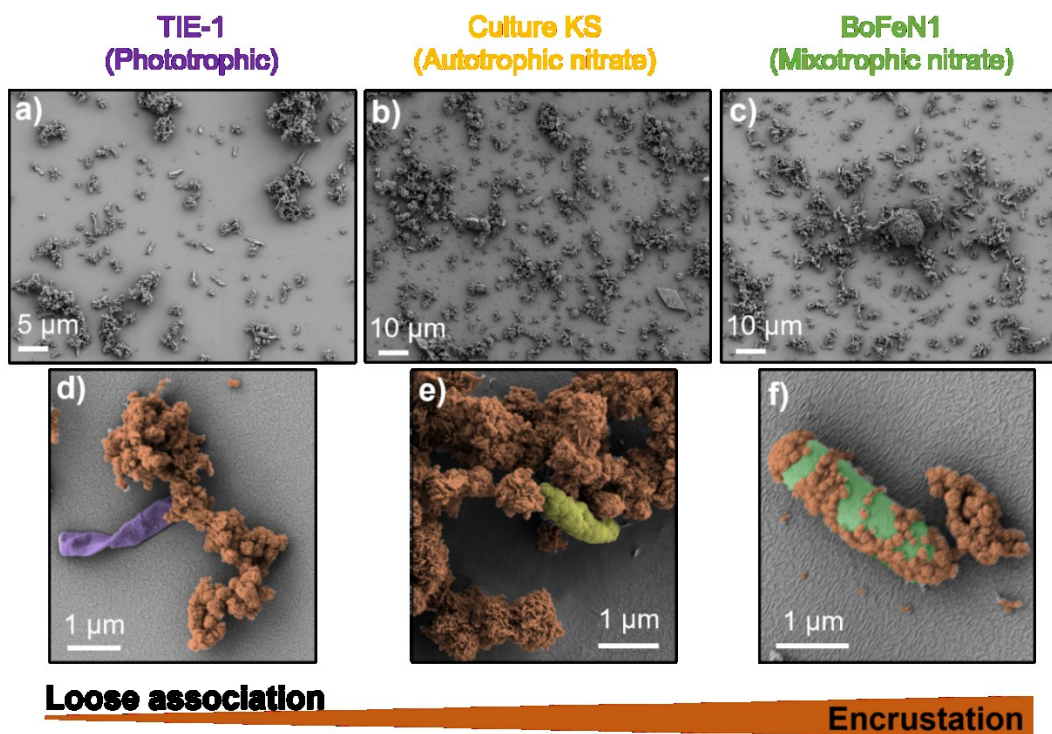


Figure. 1. SEM micrographs of BIOS used in this study. Overview of (a) TIE-1 (b) KS and (c) BoFeN1. Close up false color images show that (d) TIE-1 (purple) has loose cell-mineral associations. (e) KS (yellow) has partial associations with some cells of the flanking community being encrusted. In the image, one representative fully encrusted cell, with characteristic granular surface made by the precipitated Fe(III) (oxyhydr)oxides is shown. (f) BoFeN1 (green) has high degree of encrustation.

Particle size distribution, specific surface area and zeta potential

The specific surface area (SSA) of the BIOS ranged from 75.1 to 144.4 m²/g, with the highest value observed for TIE-1 (**Table 2**). The particulate size distribution of unfiltered BIOS in this study ranged from hundreds of nm to a few hundred μ m (up to ~2,000 μ m for TIE-1). In the case of TIE-1, we observed two size fractions, one with a mean diameter of 23 μ m and another one with a mean diameter of 1553 μ m (**Table 2**). These are larger compared to BoFeN1 (12.8 ± 1.4 μ m) and KS (7.9 ± 0.3 μ m).

Table 2. Mean particulate size (μ m), specific surface area (SSA), total organic carbon (TOC), zeta potential (mV) and mean colloidal size (nm) of BIOS, original and after freeze-thawing, with additional values from the literature for Thiodictyon sp. strain F4 (F4), *R. ferrooxidans* sp. strain SW2 (SW2) and Gallionella-like environmental BIOS from groundwater (GL). Grey values indicate the smaller absolute value when comparing between original and freeze-thawed BIOS.

	TIE-1		BoFeN1		KS		F4	SW2	GL
	Original	Freeze-thawed	Original	Freeze-thawed	Original	Freeze-thawed			
Mean particulate size (μ m)	23; 1553	23.8 $\pm$ 0.8; 209 ^d	12.8 $\pm$ 1.4 ^d	17.4 $\pm$ 0.0 ^a	7.9 $\pm$ 0.3 ^d	138	10-40 ^a	10-40 ^a	
SSA (m ² /g)	144.4	98.4	80.4 158 ^a	180.5	75.1	55.2			
TOC (wt%)	2.2 $\pm$ 0.2	1.5 $\pm$ 0.03	1.3 $\pm$ 0.0	1.3 $\pm$ 0.0	0.24 $\pm$ 0.01	0.16 $\pm$ 0.001		[C] $\equiv$ ([Fe]-1.2 ^c 100)/2.5 ^b	
Zeta potential (mV)	-3.35 $\pm$ 0.35 ^d	-8.00 $\pm$ 1.0 ^e	-3.15 $\pm$ 0.25 ^d	-8.45 $\pm$ 0.55 ^e	5.40 $\pm$ 1.20 -4.60 $\pm$ 2.30	-16.10 $\pm$ 1.10 ^e			
Mean colloidal size (nm)	1087.5 $\pm$ 186 ^d	290 $\pm$ 7 ^d	550 $\pm$ 28 ^d	491 $\pm$ 48 ^d	675 $\pm$ 130 ^d	833 $\pm$ 101 ^e			

^a (Posth et al., 2010)

^b Relationship of Fe-C concentration in the extracellular filaments of the photoferrotroph *Rhodobacter* sp. strain SW2 as reported by (Miot et al., 2009)

^c (Cismasu et al., 2016)

^d Replicate bottles = 2

^e Replicate bottles = 3

We also analyzed colloidal Fe(III) oxyhydroxides (fractions less than 1.2 μ m in size), as these are typically the most reactive fractions. Ferrozine analysis showed that this fraction constituted less than 5% of the total Fe. The colloidal mean size was 1088 $\pm$ 186 nm, 675 $\pm$ 130 nm and 550 $\pm$ 28 nm for TIE-1, KS, and BoFeN1, respectively. Their zeta potentials were mostly negative (-1 to -5.3 mV, with one outlier at 5.4 mV), consistent with the presence of organics on the surface of the Fe(III) minerals (**Table 2**).

Freeze-thawing led to changes in their physiochemical parameters. It led to consistently more negative zeta potential (-17 to -7 mV across all 3 FeOB). The effect on particle size and SSA were FeOB-specific. For SSA, that of BoFeN1 increased to 180.5 m²/g, while it decreased to 98.4 and 55.2 m²/g in TIE-1 and KS, respectively. For particulate size distribution of unfiltered samples, the mean size of the smaller size group of TIE-1 BIOS remained unchanged, but the larger aggregates (1553 µm) decreased to an average size of 209 µm. Meanwhile, the mean size of both BoFeN1 and KS particulates increased to 17.4 ± 0.0 µm and 138 µm, respectively (**Fig. S4**). Finally, the colloidal size distribution decreased after freeze-thawing for TIE-1 (290 ± 7 nm) and KS (833 ± 101 nm), while it increased for BoFeN1 (491 ± 48 nm).

Carbon content in the solid phase

The TOC in the solid-phase fraction of BIOS – corresponding to EPS and cells remaining after washing – is reported in **Table 2**. TIE-1 has the highest TOC (2.2%), followed by BoFeN1 (1.3%) and KS (0.24%). In our study, we found that freeze-thawing decreased significantly ($n = 3$ for original, $n = 2$ for freeze-thawed, $\alpha = 0.05$) the TOC in the BIOS of TIE-1 to 1.5% and KS to 0.16%, while TOC in the BIOS of BoFeN1 remained unchanged (1.3%). Due to the low concentration of TOC across all setups, the characteristic bacterial FTIR spectrum was overwhelmed by bands that can be attributed to Fe(III) minerals (data not shown).

BIOS as electron donor and acceptor

We incubated the FeRB, *S. oneidensis* and *G. sulfurreducens* without the addition of bioavailable OC sources, to test the bioavailability of BIOS as both electron donor and acceptor to examine their bioavailability. In the negative controls, the Fe(II)/Fe(total) ratio remained at 6.9 ± 2.3%, 15.4 ± 4.9% and 9.9 ± 4.5% with original BIOS of TIE-1, BoFeN1 and KS, respectively. At the end of the reduction experiment, the Fe(II)/Fe(total) ratio reached a maximum of 52% (**Fig. 2**). BoFeN1 BIOS were the most bioavailable, with a higher Fe(III) reduction extent with both FeRB, followed by TIE-1 and finally KS. BIOS of BoFeN1, TIE-1 and KS were reduced to 52%, 44% and 36% by *G. sulfurreducens*, respectively; and up to 43, 34 and 31% for BIOS of BoFeN1, TIE-1 and KS by *S. oneidensis*, respectively (**Table S2**). In the setup with *G. sulfurreducens*, the maximum extent was reached within 10 days, at a rate of 0.16, 0.41 and 0.23 mM Fe (II)/day for BIOS of TIE-1, BoFeN1 and KS respectively (**Fig. 2a; Table S2**). *S. oneidensis* reduced BIOS_{Fe(III)} in 40 days, with a lower rate of 0.09, 0.05 and 0.08 mM Fe(II)/day for BIOS of TIE-1, BoFeN1 and KS, respectively (**Fig. 2b; Table S2**). Note, however, that despite the absence of additional OC in the

setups, the preculture carried leftover OC (e.g., acetate, lactate) that probably served as electron donor (see Discussion, section *Bioavailable OC controls the extent of BIOS Fe(III) reduction*).

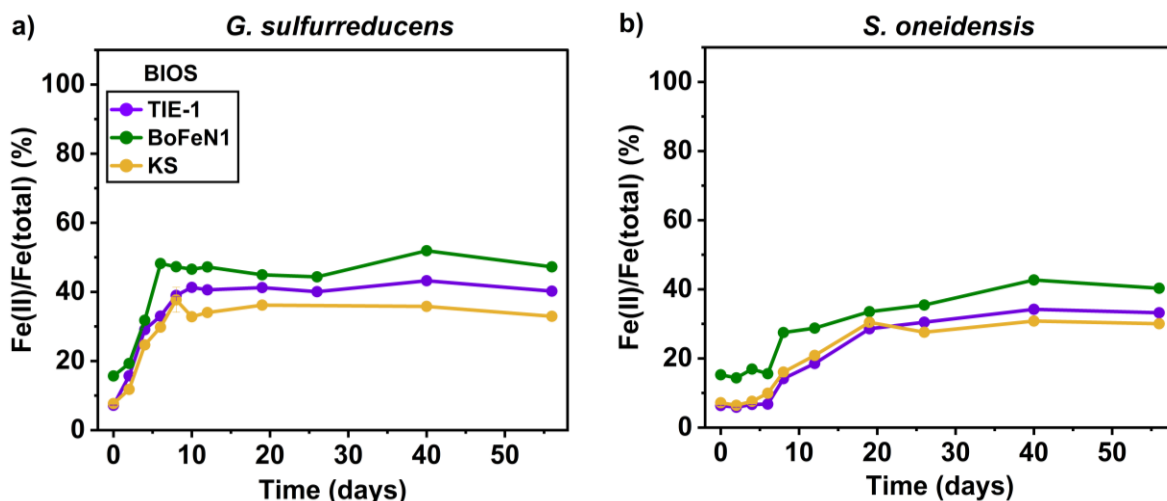


Figure 2. Fe(II)/Fe(total) ratio of the reduction of ~6 mM BIOS_{Fe(III)} by (a) *G. sulfurreducens* and (b) *S. oneidensis* without additional acetate or lactate. Data shown are the mean of experimental triplicates, error bars that indicate the standard deviation ($n = 3$) are smaller than the symbols in the figures.

Fe(III) reduction in the presence of available electron donors

To investigate if organic carbon was the factor limiting BIOS_{Fe(III)} reduction, 5 mM lactate or acetate, respectively, in excess, were introduced into the setups as an electron donor. In the negative control with lactate, the Fe(II)/Fe(total) ratio of original BIOS of TIE-1, BoFeN1, and KS remained at $6.3 \pm 1.8\%$, $15.6 \pm 4.9\%$, and $7.3 \pm 1.9\%$, respectively. In the negative control with acetate, the Fe(II)/Fe(total) ratio of BIOS of TIE-1, BoFeN1 and KS remained at $6.8 \pm 1.1\%$, $18.3 \pm 4.0\%$ and $8.0 \pm 2.9\%$, respectively. Both strains were able to reduce 100% of the BIOS_{Fe(III)} in all cases. *G. sulfurreducens* reduced the BIOS significantly ($n = 3$, $\alpha = 0.05$) faster than *S. oneidensis*, reducing them within 12 days with a rate of 0.72, 0.69 and 0.44 mM Fe (III)/day for TIE-1, BoFeN1 and KS BIOS, respectively (**Fig. 3a; Table S3**). *S. oneidensis* took longer to reduce the BIOS_{Fe(III)} (within 40 days) with a rate of 0.13, 0.12 and 0.15 mM Fe(III)/day for TIE-1, BoFeN1 and KS BIOS, respectively (**Fig. 3b; Table S3**). In these setups, we also performed additional incubations with synthetic ferrihydrite as the Fe(III) source. For both FeRB, we observed a much slower reduction rate of ferrihydrite relative to the BIOS, and similar between the FeRB, 0.07 ± 0.01 and 0.08 ± 0.00 mM Fe(III)/day with *G. sulfurreducens* and *S. oneidensis*, respectively (**Fig. 3; Table S3**).

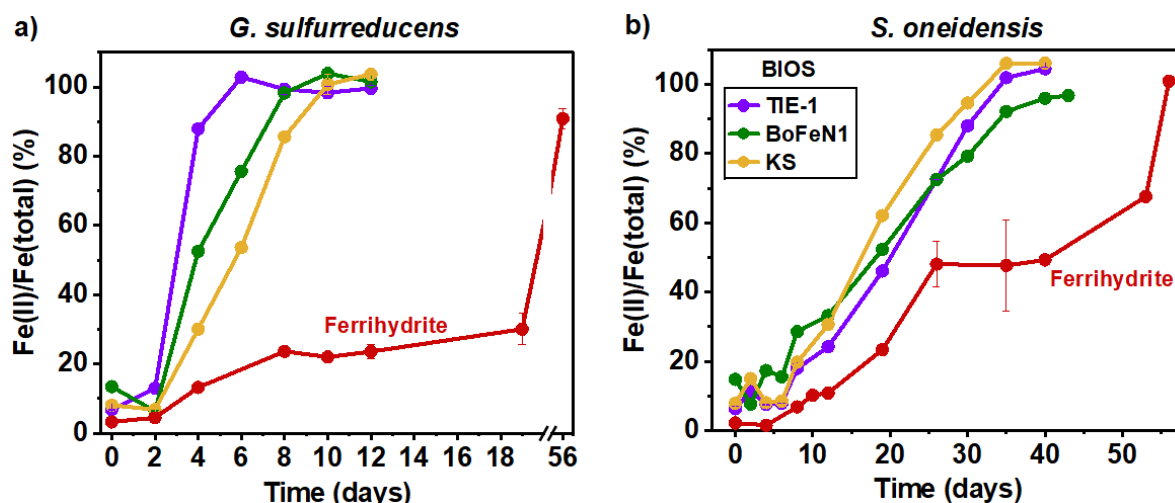


Figure 3. Fe(II)/Fe(total) ratio of the reduction of ~6 mM BIOS_{Fe(III)} (mixture of ferrihydrite, goethite and lepidocrocite) and synthetic 2-line ferrihydrite by (a) *G. sulfurreducens* and (b) *S. oneidensis* in the presence of 5 mM of acetate or lactate, respectively. Shown are the mean of experimental triplicates for BIOS and duplicates for ferrihydrite; error bars (in most cases are smaller than and hidden by the symbols) indicate the standard deviation ($n = 3$).

Effect of freeze-thawing on Fe(III) reduction

We tested the impact of freeze-thawing on the bioavailability of BIOS. The results showed that the Fe(II)/Fe(total) ratio in negative controls remained $9.4 \pm 4.0\%$, $15.6 \pm 9.1\%$, and $28.3 \pm 8.6\%$ with freeze-thawed BIOS of TIE-1, BoFeN1, and KS, respectively, without any additional electron donor. *G. sulfurreducens* reached a maximum BIOS_{Fe(III)} reduction extent of 49%, 40% and 40% for BIOS of KS, BoFeN1 and TIE-1, respectively (**Table S2**), within 61 days (**Fig. 4a**). Within the same period, *S. oneidensis* reached a maximum BIOS_{Fe(III)} reduction extent of 43%, 25% and 25% for BIOS of KS, BoFeN1 and TIE-1, respectively (**Fig. 4b**). In all setups, the Fe(III) reduction rates between the two FeRB were statistically different ($n = 3$, $\alpha = 0.05$). *G. sulfurreducens* was faster at reducing BIOS_{Fe(III)} from TIE-1, BoFeN1 and KS at 0.09 ± 0.02 , 0.06 ± 0.01 and 0.29 ± 0.06 mM Fe(III)/day, respectively, while *S. oneidensis* reduced them at 0.03 ± 0.01 , 0.04 ± 0.02 , and 0.24 ± 0.06 mM Fe(III)/day, respectively (**Table S3**).

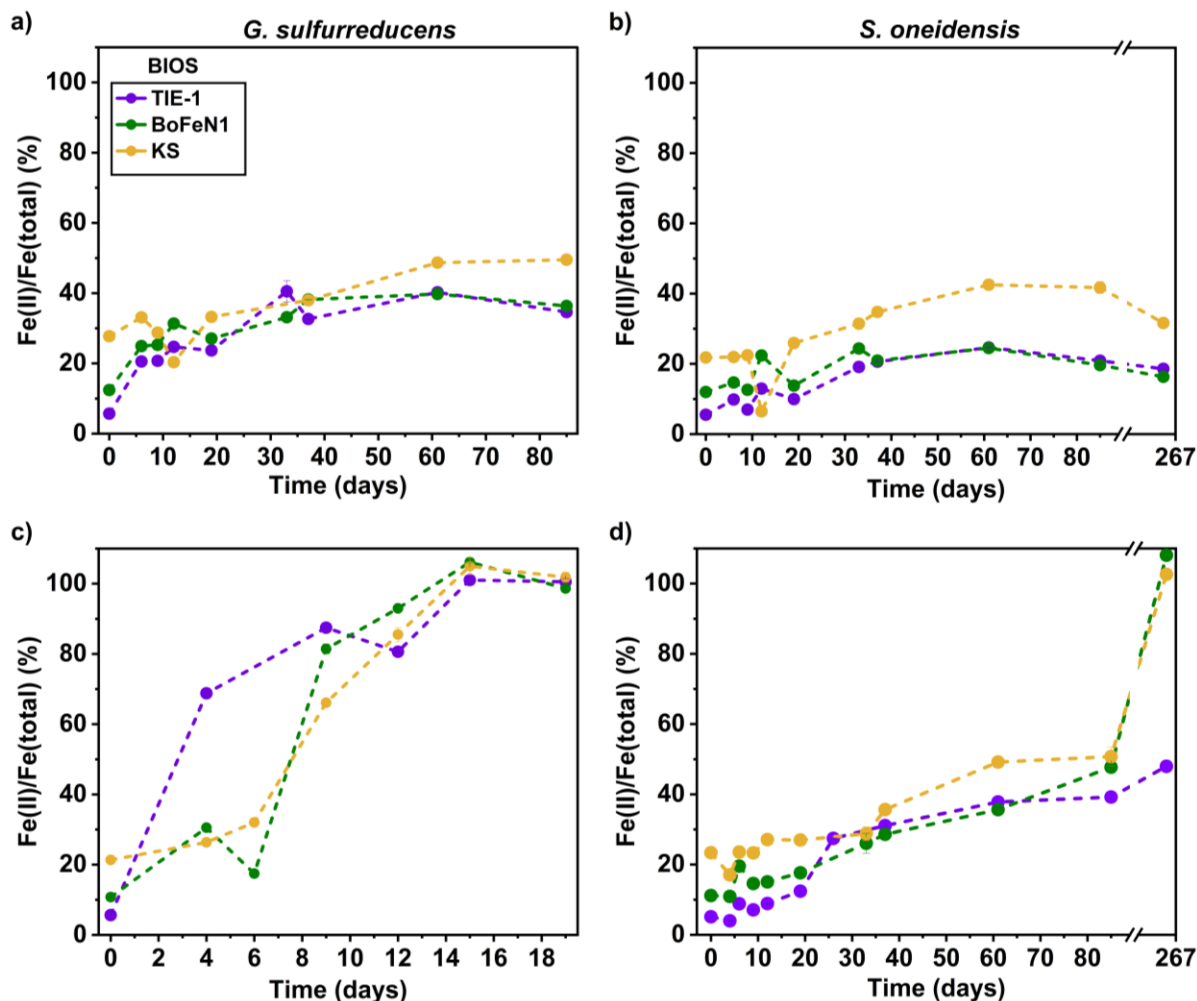


Figure 4. Fe(II)/Fe(total) ratio of freeze-thawed BIOS_{Fe(III)} reduction by (a) *G. sulfurreducens* and (b) *S. oneidensis* without additional carbon and in the presence of 5 mM of acetate and lactate for (c) *G. sulfurreducens* and (d) *S. oneidensis*, respectively. Data shown are the mean of experimental triplicates for BIOS and error bars (in most cases hidden by the symbols) indicate the standard deviation.

With additional acetate, the Fe(II)/Fe(total) ratio in negative controls remained 9.1 ± 4.0 , 16.6 ± 8.2 and 27.4 ± 5.1 mM Fe(II) with freeze-thawed BIOS of TIE-1, BoFeN1 and KS, respectively. *G. sulfurreducens* reduced 100% of the freeze-thawed BIOS_{Fe(III)} in 15 days (**Fig. 4c**). With lactate, the Fe(II)/Fe(total) ratio in negative controls remained 9.4 ± 4.4 , 16.4 ± 7.3 and 29.4 ± 8.9 mM with freeze-thawed BIOS of TIE-1, BoFeN1 and KS, respectively. Despite the addition of lactate, after 85 days *S. oneidensis* showed a reduction extent of only 51%, 48% and 39% for BIOS of KS, BoFeN1 and TIE-1, respectively. After 267 days, an additional sampling showed that KS and

BoFeN1 BIOS had been fully reduced by *S. oneidensis*, while TIE-1 BIOS only had a maximum reduction extent of 48% (**Fig. 4d**).

When comparing to untreated BIOS, freeze-thawing tended to decrease the BIOS_{Fe(III)} reduction rates (3 out of 6 conditions) and extents (4 out of 6 cases) in the absence of added OC. In the presence of added OC, the reduction rates tended to be lower (5 out of 6 conditions) while the extents tended to be comparable due to the reaction reaching completion (5 out of 6 conditions) (**Table S2 and S3**). The overall decrease in reduction rates ranged from 1.5-10-fold lower compared to untreated BIOS. The overall decrease in reduction extent typically ranged from 10-20% compared to untreated BIOS, with one outlier exhibiting a 52% decrease (**Table S2, S3, S4**).

Discussion

Properties of BIOS compared to literature

Fe(III) (oxyhydr)oxides are important sinks and sources of biogeochemically important nutrients. They are known carbon sinks, trapping a substantial amount of OC in terrestrial (~40%) and marine (~19%) sediments (Jia et al., 2024; Lalonde et al., 2012). Extensive work has been done to study the mineral composition of BIOS and their biomineralization patterns, particularly on the phenomenon of encrustation that occurs in some nitrate-reducing Fe(II)-oxidizing bacteria (**Fig. 1**). Despite this, information on their basic properties (e.g., surface areas, zeta potential, particle size distribution, TOC) is lacking. Our work adds to this incomplete body of literature and allows comparison with previous datasets.

The SSA of BIOS has been determined only once before, yielding a value of 158 m²/g for BoFeN1 (reported in Posth et al., 2010, although without reference to the original paper). We measured SSA with a range of 75-144 m²/g for three types of BIOS, with a value of 80 m²/g specifically for BoFeN1. While the SSA values are generally high and reflect their poorly crystalline nature (Song et al. 2022), it seems that differences in growth conditions or culture batch could yield variation in SSA of BIOS from the same species (**Table 2**).

TIE-1 displayed the highest TOC (2.2 wt%), followed by BoFeN1 (1.3%) and KS (0.24%). TIE-1 is a photoferrotroph, which are known to secrete EPS to prevent encrustation (Wu et al. 2014). In BoFeN1, cells are typically encrusted with Fe(III) oxyhydroxides, resulting in tight OC association within BIOS (Kappler et al. 2005; Schmid et al. 2014). Finally, culture KS is an autotrophic nitrate-reducing Fe(II)-oxidizing co-culture, in which any OC needs to be sourced from energetically

expensive fixation of CO₂. Hence, patterns in the TOC content are broadly consistent with their metabolisms. In comparison to the literature, the Fe-C content within the extracellular filaments of the photoferrotroph *Rhodobacter* sp. strain SW2 was determined to follow the relationship $[Fe]=2.5[C]+100$ (Miot et al., 2009), while ~1.2% TOC was determined in *Gallionella*-like BIOS from groundwater (Cismasu et al., 2016) (**Table 2**).

In this study, we measured the particle size distribution of BIOS in suspension using a Mastersizer and a Zetasizer, revealing a broad distribution from hundreds of nm to a few hundred μm (up to ~2,000 μm for TIE-1). Previous studies have used electron microscopy to estimate the size of cell-mineral aggregates in FeOB, showing that individual particles are typically <1,000 nm (Huang et al. 2023; Jiao et al. 2005). Hence, the large size ranges we measured signify the formation of aggregates composed of minerals, cells, and/or EPS in suspension. Here, the mean aggregate size for the three investigated cultures was TIE-1>BoFeN1>KS. The larger aggregates in TIE-1 could be induced by EPS that act as binding agents, produced by the cells to prevent cell encrustation by minerals (Wu et al., 2014). In comparison, other photoferrotrophs (*Thiodictyon* sp. F4 and *Rhodobacter ferrooxidans* sp. strain SW2) also produced relatively large BIOS with a size range of 10-40 μm (as measured on a Mastersizer, Posth et al., 2010) that were larger than those of BoFeN1 (mean = 11-14 μm) and KS (mean = 8 μm).

The particle size analysis performed here also conclusively showed the presence of colloids <1,200 nm in suspension, although their amount was less than 5% of the total Fe. These colloids have a size range of 550-1,100 nm, dependent on the BIOS source and the exact culture bottle. The zeta potential for these colloids was predominantly negative, ranging from -1.0 to -5.3 mV, with one positive value for KS at +5.4 mV. These values reflect the influence of negatively charged OC on the surfaces of BIOS from deprotonated functional groups like carboxyl and phosphate groups (Ciempiel et al. 2025; Huang et al. 2022), as at pH 7 the zeta potential of Fe(III) (oxyhydr)oxides should be close to 0 or positive, given their point of zero charges of 7-9 (Venema et al. 1996; Mansor et al. 2025; Kosmulski 2009). Overall, colloids from BIOS are present at low concentrations and tend to be negatively charged.

Disentangling factors controlling the BIOS_{Fe(III)} reduction rate and extent

Following characterization, we tested the bioavailability of BIOS for microbial Fe(III) reduction across multiple variables relevant to natural environments, resulting in a total of 24 conditions (**Table S4**). We therefore performed a redundancy analysis to identify the most important

parameters controlling the extent and rate of BIOS_{Fe(III)} reduction (**Fig. 5**). The analysis showed that OC addition was the main factor influencing the extent of reduction. In comparison, the rate was primarily controlled by the FeRB species, with *G. sulfurreducens* being faster than *S. oneidensis* both with and without OC. Among the BIOS sources, BoFeN1 showed the highest reduction rates, both with and without OC. Finally, freeze-thawing tended to decrease the reduction rates without affecting the extent of reduction. Below, we discuss the potential mechanisms based on these trends in more detail.

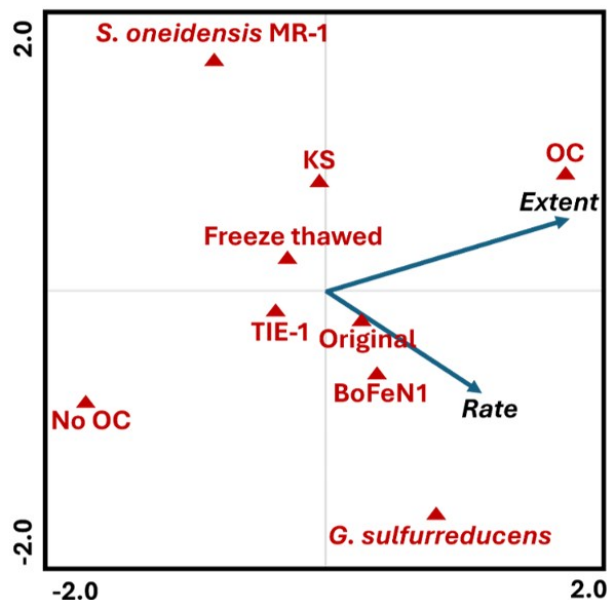


Figure 5. Redundancy analysis to determine the factors affecting the rate and extent of microbial BIOS reduction. The factors evaluated were the origin of the BIOS (TIE-1, BoFeN1, KS), the FeRB species (*G. sulfurreducens*, *S. oneidensis*), usage of additional carbon (OC, No OC) and BIOS treatment (original, freeze-thawed). Blue arrows are the response variables (rate and extent) and red triangles are the predictor variables. A list with the complete set of samples can be found in **Table S4** and **Fig. S5** displays the results including the individual samples.

Comparison of the bioavailability of BIOS with different origins and synthetic ferrihydrite

Overall, the source of the original BIOS contributed to small differences in Fe(III) reduction rates and <10% differences in reduction extent. BIOS_{Fe(III)} from BoFeN1 was the most bioavailable, followed by TIE-1 and KS. The reason(s) for these differences are unclear, as they seem to be uncorrelated with their mineralogy, particle size, TOC, SSA, and zeta potential (**Table 2**).

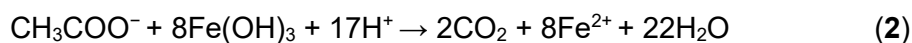
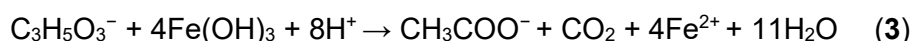
Compared to synthetic ferrihydrite, (used due to its higher reactivity compared with other Fe(III) (oxyhydr)oxides (Lovley 1991; Coker et al. 2008; Cutting et al. 2009; Han et al. 2018)), the Fe(III)

in all BIOS used here was more reactive and had higher reduction rates (8-13 and 1.5-5 times with *G. sulfurreducens* and *S. oneidensis*, respectively; **Table S3**). We attribute this higher availability to the presence of organic carbon within BIOS. The generally negative zeta potential of the BIOS suggest the presence of organic molecules on the mineral surfaces. These organics may contain redox-active species that facilitate electron transfer between the cells and the minerals. Moreover, the presence of OC could shift the redox potential towards greater reducibility. Similar interpretations have been made in the past in relation to Fe-OC coprecipitates that showed higher reduction rates and extents compared to synthetic Fe(III) (oxyhydr)oxides (Mikutta 2011; Poggenburg et al. 2016).

We also considered two additional factors: mineralogy and surface areas. Mineralogy-wise, since BIOS contained goethite, we would expect it should have a lower bioavailability compared to synthetic ferrihydrite. Similarly, as ferrihydrite typically has a very high surface area (>200-400 m² g⁻¹; He et al. 2025; Macera et al. 2020; Brinza 2022), we would expect BIOS to be less bioavailable compared to ferrihydrite. Hence, it seems that despite these factors, the presence of redox-active species covering the minerals in BIOS results in a stronger effect with higher reduction rates. This concurs with the previously demonstrated higher bioavailability of BIOS_{Fe(III)} from environmental samples compared to ferrihydrite, with BIOS being reduced 1.5-3 times faster than ferrihydrite (Langley et al., 2009). Our results conclusively show the high bioavailability of BIOS for microbial Fe(III) reduction. They further highlight the importance of using environmentally relevant materials such as BIOS instead of purely synthetic minerals to derive reduction rates and extents relevant to nature.

Bioavailable OC controls the extent of BIOS_{Fe(III)} reduction

In setups where *G. sulfurreducens* and *S. oneidensis* were incubated with only BIOS, BIOS_{Fe(III)} reduction was incomplete, with a maximum extent of 52% (of 6 mM Fe(III)) across all BIOS. The BIOS_{Fe(III)} reduction in these setups is attributed to the small yet present OC transferred from the precultures. In the case of *G. sulfurreducens*, due to the absence of washing, ~0.17 mM of acetate could be transferred from the preculture, enough to reduce ~1.36 mM of Fe, which is consistent with the ~2.0 mM of Fe(II) produced (**Eq. 2**) (considering this includes ~0.61 mM of initial Fe(II)). Likewise, *S. oneidensis* may contain ~1 mM of lactate coming from the preculture, enough to reduce 4 mM of Fe, which is higher than the ~1.9 mM of Fe(II) produced (**Eq. 3**).



When incubation was performed in the presence of excess of electron donor by adding acetate/lactate, all the BIOS_{Fe(III)} was reduced (**Fig. 3**), which confirms the high bioavailability of BIOS_{Fe(III)}. This result also highlights the limited bioavailability of BIOS-associated organic carbon (BIOS_{OC}) to *G. sulfurreducens* and *S. oneidensis*. BIOS_{OC} could have been inaccessible due to the complexity of the organic molecules, strong chemical bonding between Fe and C, or physical inhibition by the Fe(III) oxyhydroxides. In fact, neither of the FeRB is known to degrade high-molecular-weight molecules, apart from reducing disulfide bonds in organic matter (Phan et al., 2024; Voordeckers et al., 2010). In the presence of EPS – a main component of BIOS_{OC} – Fe(III) oxyhydroxides displayed lower reduction rates than with natural organic matter (Cooper et al., 2017; Gao et al., 2019; Poggenburg et al., 2018). Furthermore, due to aggregation, organic carbon could be trapped within aggregate pores, making it less accessible to degradation. These combined factors contribute to limited bioavailability of BIOS_{OC} to the two model FeRB species we tested here. The bioavailability of BIOS_{OC} to other FeRB and microbial species (e.g., fermenters) remains an open question.

FeRB-specific differences in BIOS_{Fe(III)} reduction

Intriguingly, *G. sulfurreducens* generally displayed higher BIOS_{Fe(III)} reduction rates compared to *S. oneidensis*. We considered two factors to account for this difference. First, as the initial cell numbers were the same (~10⁵ cells/mL), differences in the Fe(III) reduction rate cannot be explained by this parameter and suggest that *G. sulfurreducens* has a faster cell-specific reduction rate. However, we did not track differences in cell numbers as the incubation proceeded. Second, we considered differences in reduction mechanisms between the two FeRB. *G. sulfurreducens* preferentially reduces Fe(III) via direct cell-mineral contact, aided by electron transfer via conductive pili (Ueki, 2021). In contrast, *S. oneidensis* preferentially uses electron shuttles (e.g. quinone moieties), which are known to enhance the reduction of Fe(III) and allow longer electron transfer distances to the terminal electron acceptor (Chen et al., 2017; Lovley & Blunt-Harris, 1999; Meng et al., 2018). In the absence of added electron shuttles, *S. oneidensis* typically needs time in the order of 48-50 h to synthesize their own electron shuttles (e.g., flavins) (Marsili et al., 2008; Von Canstein et al., 2008) or to adapt to a direct contact mechanism (Kotloski & Gralnick, 2013; Lies et al., 2005), which may explain their longer lag phase compared to *G. sulfurreducens* in our setups.

Previous experiments have shown differing Fe(III) reduction rates of Fe-OC coprecipitates by *Shewanella* and *Geobacter* species, depending on the C/Fe ratio. Increasing C/Fe ratios led to faster Fe(III) reduction for *Shewanella*, attributed to the presence of quinone-containing molecules acting as electron shuttles. In contrast, increasing C/Fe ratios led to slower Fe(III) reduction for *Geobacter*, attributed to surface passivation effect that prevents *Geobacter* from attacking mineral surfaces (Cooper et al., 2017). Thus, organic matter can influence the Fe(III) reduction rate of different FeRB species. In comparison to our experiment, two observations are important. First, the molar C/Fe ratios of 0.02, 0.10 and 0.17 for BIOS of KS, BoFeN1 and TIE-1, respectively, are at the low end of those observed for Fe-OC coprecipitates. Even at these low C/Fe ratios, a significant enhancement of reduction was observed for both *Geobacter* and *Shewanella* compared to ferrihydrite, attributed to the presence of redox-active species that enhanced electron transfer between cells and minerals (Martinez and Alvarez 2018; Poggendorf et al. 2016). Second, at comparable C/Fe ratios, the Fe(III) reduction rates for *Geobacter* were always faster than *S. oneidensis* (Cooper et al. 2017; Eusterhues et al. 2014; Lovley 1991). Our results therefore are consistent with previous studies and further highlight the higher Fe(III) reduction rates of *Geobacter* compared to *Shewanella* species.

Effect of freeze-thawing on the properties of BIOS

Freeze-thawing is a process relevant to high-latitude zones, mountain glaciers, and other temperate climate regions across Earth (Hayashi, 2013). As Fe-cycling bacteria are active in these environments, it is imperative to understand the effects of freeze-thawing on the properties and bioavailability of BIOS (Nixon et al. 2017; Laufer-Meiser et al. 2021; Michaud et al. 2023; Toubes-Rodrigo et al. 2021). We found the effect of freeze-thawing to be complex and dependent on multiple parameters. For example, freeze-thawing consistently decreased the surface charges of colloidal BIOS regardless of origins. We speculate that this is due to the breakdown of complex organic molecules (e.g., cells and EPS) during freezing (Fellman et al., 2008; Pokrovsky et al., 2018) followed by their migration to the outer surface of the BIOS aggregates upon thawing (Gu et al. 1995). This process did not lead to changes in the TOC of BIOS of BoFeN1, while significantly ($\alpha=0.05$; $n=3$) decreasing the TOC content of TIE-1 and KS BIOS.

Freezing likely led to changes in the BIOS structure, as inferred from changes in SSA and particle size distribution. In the case of TIE-1, the decrease in SSA and particle size indicated freezing-induced compaction. For BoFeN1, the increase in SSA and particle size were more consistent with aggregation into an open structure with high accessibility to reactive sites. In the case of KS,

the decrease in SSA coupled with an increase in particle size distribution implied aggregation to larger structures with limited access to reactive surfaces. We note, however, that these are only interpretations, as the Mastersizer and Zetasizer only measure the hydrodynamic diameter, *i.e.*, it assumes a hypothetical hard sphere in solution, including its surface structure and solvation layer, without giving insight into the 3D arrangement or geometric size, *i.e.*, the actual shape and geometry, of the BIOS after freeze-thawing.

Lastly, freezing did not cause a change in mineralogy nor a large difference in crystallinity of the BIOS. Freeze-thawing also potentially caused a slight increase from a Fe(II)/Fe(total) ratio of $6.9 \pm 0.6\%$, $13.4 \pm 0.3\%$, and $27.1 \pm 0.5\%$, to $9.3 \pm 4.2\%$, $16.6 \pm 7.8\%$, and $28.2 \pm 7.5\%$ for BIOS of TIE-1, BoFeN1, and KS, respectively, although the values still overlap if we consider the high standard deviations.

Effect of freeze-thawing on the bioavailability of BIOS

In general, freeze-thawing tended to decrease the bioavailability of BIOS_{Fe(III)}, although the effect is specific to the variables tested. One potential reason is the more negative surface charge that developed on BIOS upon freezing. Since microbial cell membranes are generally negative, accessing the surface of BIOS via direct contact becomes more difficult when electrostatic repulsion needs to be overcome (Deville, 2017; Trinks et al., 2005).

To gain additional insight into the effect of freeze-thawing on bioavailability, we first considered setups in which additional labile OC was added. In most cases, the reduction extents reached 100%, highlighting the high bioavailability of BIOS_{Fe(III)} even after freeze-thawing. The one exception is for TIE-1 BIOS reduced by *S. oneidensis* in which case the reaction stopped at 48% reduction extent – we consider this to be an outlier. In 5 out of 6 conditions, the reduction rates were lower after freeze-thawing. This emphasizes the lower accessibility of BIOS upon freeze-thawing. The one exception was for BoFeN1 BIOS reduced by *G. sulfurreducens*, which exhibited a higher rate after freeze-thawing. This is perhaps related to the difference in the reduction mechanism of the respective FeRB and their response to the spatial Fe-OC association within BIOS upon freeze-thawing.

Next, we consider cases in which no labile OC was added. Here, TIE-1 BIOS displayed lower reduction rates and extents with both FeRB. This is consistent with the expectation of lower bioavailability due to decreased SSA and particle size after freeze-thawing. In comparison, BoFeN1

BIOS displayed lower or comparable reduction rates after freeze-thawing. This is not expected based on its increase in SSA, but is consistent with its increase in particle size that may have led to lower accessibility to the FeRB after freeze-thawing. In contrast to the other two BIOS, KS BIOS displayed higher reduction rate and extent after freeze-thawing. This is surprising considering the decrease in SSA and increase in particle size after freeze-thawing. Overall, our results suggest that SSA and particle size are poor predictors of relative reduction rate and extent after freeze-thawing treatment. This highlights the necessity of performing microbial reduction experiments to directly measure bioavailability in the presence of multiple completing variables.

Conclusion

As the transformation of Fe minerals interlinks with other elemental cycles (e.g., C, N, and P), here we investigated the bioavailability of BIOS from FeOB. We tested multiple variables relevant across different environments: three different origins of BIOS, two model FeRB, with and without organic carbon, and before and after freeze-thawing. Based on RDA analysis, our study shows that OC availability is the primary factor controlling the bioavailability of BIOS_{Fe(III)}. In the presence of excess labile OC, BIOS_{Fe(III)} can be completely reduced, exhibiting higher reduction rates compared to synthetic ferrihydrite. This is even more surprising considering that BIOS are composed of mixtures of ferrihydrite with more crystalline lepidocrocite and goethite. Hence, it emphasizes the need to conduct experiments with naturally relevant materials to avoid underestimation of reaction rates relevant to the environment.

In soils that undergo freeze-thawing, such as high-altitude zones or mountain glaciers, Fe-organic matter associations and the physical properties of the minerals are potentially altered, leading to different bioavailability of the minerals and OC. The impact of freeze-thawing in BIOS properties (particle size distribution, SSA, and TOC) was FeOB-specific. Only the effect on their zeta potential was consistent, increasing their magnitude towards more negative values in all BIOS. These changes in the properties had a greater impact on the rate than on the extent of reduction. Overall, freeze-thawing was a secondary control on bioavailability, with the primary factor being the presence of labile OC.

Our study also indicated limited bioavailability of the BIOS_{OC} for FeRB, although we cannot exclude its bioavailability in natural systems, where other microorganisms (e.g., fermentative microorganisms) could degrade and transform the associated OC to more bioavailable low-molecular-weight organic molecules. We leave this open question for further studies. We attempted to

directly track if any BIOS_{OC} was released to the solution. However, the low TOC content of BIOS (0.24-2.2 wt%) made this challenging. Conversion to OC/Fe molar ratios revealed a range of 0.02-0.17, which is much lower than observed for natural Fe-OC aggregates, ranging from <1.0 for sorption to a maximum of 6-10 for coprecipitation or chelation (Lalonde et al., 2012). Then, the role of BIOS on the fate of carbon is limited and linked to the carbon that could potentially adsorb and co-precipitate within BIOS, rather than to the amount of native BIOS_{OC}. We stressed however that we exclusively worked with anaerobic FeOB in this study. Environmental BIOS collected under microoxic conditions have been found to contain up to 34.5 wt% organic matter (Martinez et al. 2003). Furthermore, the higher reactivity of BIOS compared to ferrihydrite indicates that OC associated to BIOS are more likely to be released. To unequivocally trace which molecules are derived from BIOS and determine their use, other more sensitive approaches need to be undertaken in future studies, such as ¹³C labeling during BIOS formation by Fe(II)-oxidizing bacteria.

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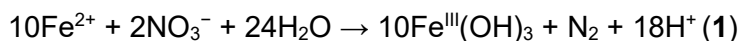
Supplementary material for: Bioavailability of biogenic iron(III) oxyhydroxides for Fe(III)-reducing bacteria

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Text S1: Metabolisms of the Fe(II)-oxidizing bacteria (FeOB) used in this study to obtain the BIOS

Acidovorax sp. BoFeN1 is a mixotrophic nitrate-reducing Fe(II)-oxidizing bacterium (NRFeOB). It was isolated from Lake Constance littoral sediments and gains energy from the enzymatic oxidation of both Fe(II) and OC (Kappler et al., 2005; Dopffel et al., 2021), oxidizing Fe(II) extracellularly with c-type cytochromes (T. Liu et al., 2019) (**Eq. 1**). The resulting Fe(III) biominerals encrust the cells even though they secrete extracellular polymeric substances (EPS) to avoid it (Klueglein et al., 2014). Encrustation occurs from the periplasm to outer membrane, until the periplasm is filled and the minerals grow into the cytoplasm after several weeks to months (Klueglein et al., 2014; Li et al., 2013; Miot et al., 2011).

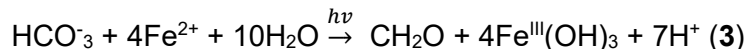


Enrichment culture KS was originally isolated from freshwater sediment samples from town ditches (Bremen, Germany) (Straub et al., 1996). The enrichment is dominated by *Ferrigenium straubiae* (>98%), an autotrophic microaerophilic Fe(II)-oxidizer bacterium (Becker & Kappler, 2025), and the main flanking microbial communities are *Rhodanobacter* and *Bradyrhizobium* (Huang et al., 2021). The microbial species function together with *Ferrigenium straubiae* fixing CO₂ via the energy gained from Fe(II) oxidation coupled to NO₃ reduction (**Eq. 1, Eq. 2**). *Rhodanobacter* sp. and *Bradyrhizobium* sp. then obtain OC that is used to reduce NO, alleviating its toxicity effect. The enrichment culture can grow in autotrophic conditions (with Fe(II) and nitrate) or in heterotrophic conditions (with acetate and nitrate) (Tominski et al., 2018). Here, we grow it under autotrophic conditions.



Rhodopseudomonas palustris TIE-1 is a purple non-sulfur bacterium isolated from an iron-rich mat from School Street Marsh in Woods Hole, MA (Jiao et al., 2005). It is a photoautotrophic

Fe(II)-oxidizer (photoferrotroph, couples CO₂ fixation to Fe(II) oxidation) (**Eq. S3**). It oxidizes Fe(II) enzymatically at the surface of the cell membrane and then transfers the electrons to the photo-reaction center in the inner cytoplasmic membrane (Shi et al., 2007). The decaheme c-type cytochrome for Fe(II) oxidation is encoded by the *pio* gene cluster (Jiao & Newman, 2007).



Cell-mineral associations

SEM images of TIE-1, BoFeN1 and KS can be seen in **Fig. 1**, with colors referring to the color code used in the figures thereafter (purple for TIE-1, green for BoFeN1 and yellow for KS). As expected, Fe(II) oxidation did not result in encrustation of TIE-1 cells (Jiao et al., 2005; Nikeleit et al., 2024), while BoFeN1 displayed partial to extensive encrustation in most cells (Kappler et al., 2005; Klueglein et al., 2014; Miot et al., 2015; Nordhoff et al., 2017; Schmid et al., 2014). In the case of KS, the encrustation depends on the Fe(II) concentration, and the flanking heterotrophic nitrate-reducers are most susceptible to encrustation (Huang et al., 2023; Nordhoff et al., 2017). With the growth conditions in this study (10 mM Fe(II) and 2 mM NO₃⁻), there was encrustation of some of the cells of the flanking community (e.g., representative cell in **Fig. 1**).

Text S2: Selection of the Fe(III)-reducing bacteria (FeRB)

Shewanella oneidensis MR-1 and *Geobacter sulfurreducens* PCA were used due to the fundamental differences in their primary reduction mechanisms (electron shuttles vs. direct cell-mineral contact, respectively).

S. oneidensis is a mesophilic, facultative anaerobe first isolated from sediments of Lake Oneida in New York State (Myers & Nealson, 1988). It can ferment or utilize diverse electron acceptors including oxygen, elemental sulfur and metal oxides (Pinchuk et al., 2011; Saffarini et al., 2015) to which it transfers the electrons extracellularly with multi-heme c-type cytochromes (Breuer et al., 2015; Hernandez & Newman, 2001). These cytochromes facilitate the transport of electrons through extracellular appendages (Pirbadian et al., 2014). It can also utilize electron shuttles such as redox-active organic matter (e.g., AQDS) or self-made flavins to facilitate extracellular electron transfer (Marsili et al., 2008).

G. sulfurreducens is an anaerobe first isolated from cores of a hydrocarbon-contaminated ditch collected near Norman, Oklahoma (Caccavo et al., 1994; Tabares et al., 2020). In *G. sulfurreducens* the electron transfer also depends on multiple *c*-type cytochromes. However, the cytochromes are mainly in the outer membrane, while those of *S. oneidensis* MR-1 are also located in the inner layer and the periplasm. Moreover, the transfer is carried out through a type IV pilus while in *S. oneidensis* the cytochromes are in direct contact with the Fe(III) (Shi et al., 2000).

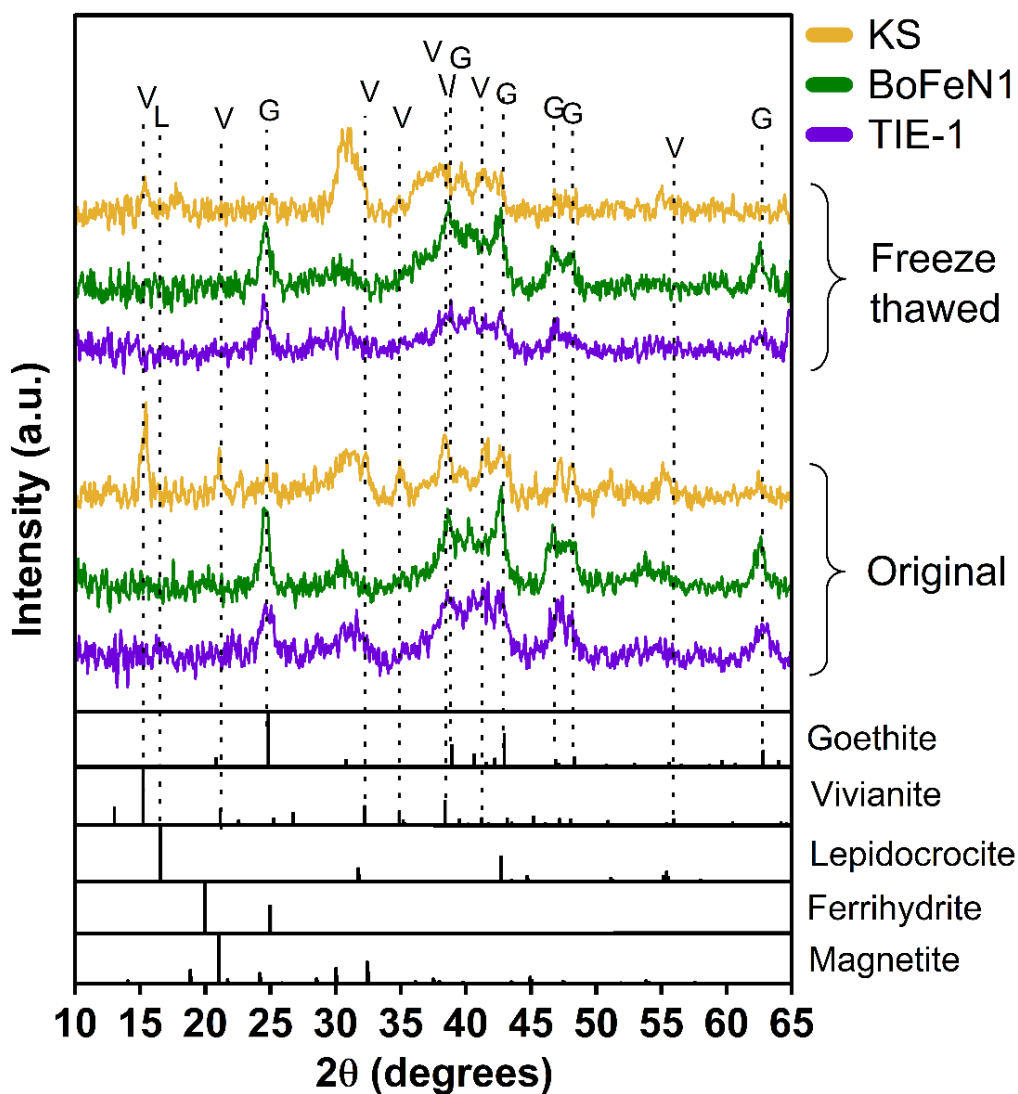


Figure S1. X-ray reflections of BIOS before (original) and after freeze-thawing (frozen). Reference patterns for goethite, vivianite, lepidocrocite, ferrihydrite and magnetite are shown at the bottom. The main crystalline mineral component of BIOS of TIE-1 and BoFeN1 is goethite. In TIE-1, lepidocrocite may be present at low quantities. In KS, goethite and vivianite are present. There is no evidence of ferrihydrite or magnetite. Reflection at 30° 2-theta did not correspond to any known iron minerals or salt.

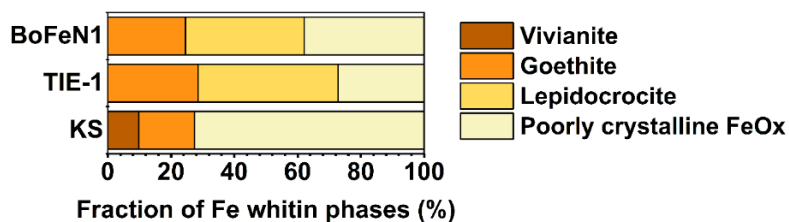


Figure S2. Relative abundance of the different Fe phases in the initial BIOS of KS, TIE-1 and BoFeN1 as determined by Mössbauer spectroscopy at 30 K. Note that the “Poorly crystalline FeOx” category includes those interpreted as ferrihydrite and Fe associated with organic matter as detailed in Table S1.

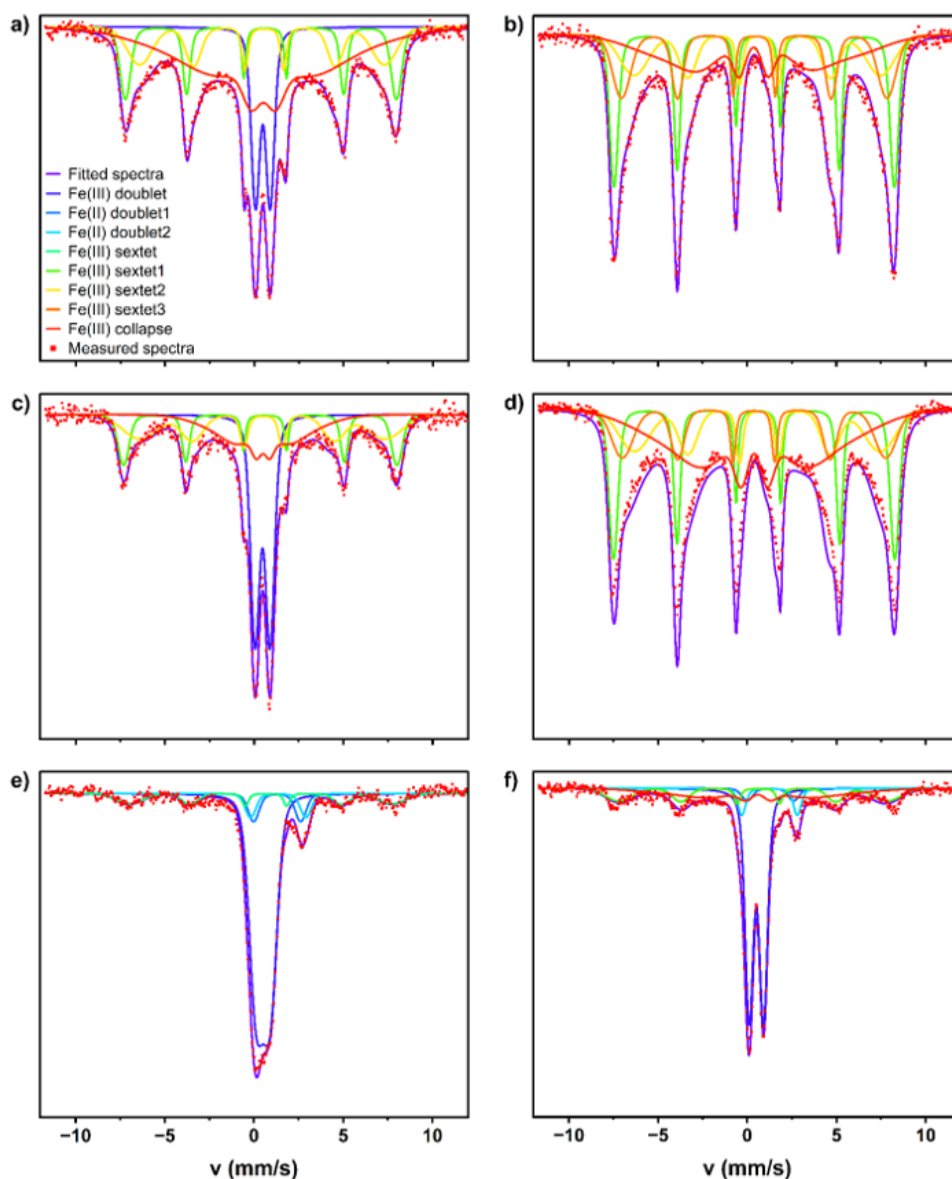


Figure S3. Mössbauer spectra of initial BIOS of TIE-1 at (a) 77 K and (b) 30 K, BoFeN1 at (c) 77 K and (d) 30 K and KS at (e) 77 K and (f) 30 K. Text S3 describes the interpretation of the spectra.

Text S3: Interpretation of the Mössbauer spectra

All initial BIOS contained a mixture of different Fe(III) oxyhydroxides, sometimes of varying crystallinities. Overall, ferrihydrite, goethite, lepidocrocite and another not fully ordered (collapsed) sextet were detected. The relative abundances of these different phases varied in each sample. Fitting parameters are shown in Table S1.

Spectra at 77 K allowed us to differentiate between poorly crystalline ferrihydrite and more crystalline minerals, like goethite and lepidocrocite. Ferrihydrite and (potentially partly) lepidocrocite are present as a doublet at 77 K and had the following parameters in the samples: center shift (CS) value of 0.47-0.51 mm/s and a quadrupole split of 0.80-0.89 mm/s (Murad & Cashion, 2011; Murad & Schwertmann, 1984). All samples contained this doublet in varying contributions (from 17% in BIOS of TIE-1 to 72% in BIOS of KS). Goethite and lepidocrocite are present as sextets and were distinguished by their hyperfine field (H) value (45.6-47.8 T and 42.5 T, respectively) and their QS (below -0.1 and -0.02 to -0.06, respectively) (Cornell & Schwertmann, 2003). All samples contained goethite. Lepidocrocite was only detected in BIOS of TIE-1 and BoFeN1. We also detected a wide doublet feature (QS > 1.3 mm/s) in the sample KS which was fit with 2 doublet components, together representative for vivianite (Dyar et al., 2014; Joëlle Kubeneck et al., 2023). The BIOS of TIE-1 and BoFeN1 also contained a collapsed Fe(III) sextet component, which was included to achieve a satisfactory fit. Since this phase is not fully ordered at 77 K, we also measured all samples at 30 K to attempt to identify this phase.

The spectra at 30 K showed another fully ordered sextet, with parameters typical for lepidocrocite: CS values of 0.40-0.49 mm/s, QS values of -0.09 to 0.02, and H values of 45.6-47.4 T (Murad & Schwertmann, 1984). However, there is still a collapsed Fe(III) sextet feature present in all samples at 30 K. We cannot fully identify this phase but it is likely a very poorly crystalline Fe(III) oxyhydroxide, possibly ferrihydrite with bound organic matter or other ions that could disturb its mineral structure. Another possibility is Fe(III)-OC complexes, although we consider this to be unlikely given the low OC concentration in the BIOS. The appearance of this collapsed sextet component coincided with the disappearance of the Fe(III) doublet (compared to the spectra of the same sample at 77 K). We speculate therefore that the Fe(III) doublet (partially) ordered to the collapsed sextet at 30 K and that the collapsed sextet at 77 K ordered to the second lepidocrocite sextet. However, with the present data it is not possible to conclude fully which phase ordered to which. The relative abundances of goethite, first lepidocrocite sextet, and vivianite were similar ($\pm 10\%$) at 30 K as in their respective spectra at 77 K.

Table S1. Fitting parameters and interpretation of different Fe phases within the initial BIOS as determined by ^{57}Fe Mössbauer spectroscopy. Abbreviations mean the following: T= temperature of measurement, CS=center shift, QS= quadrupole split in paramagnetic state or quadrupole shift, H=hyperfine field, δ_{H} = standard deviation of quadrupole split or hyperfine field, χ^2 =goodness of fit parameter.

FeOB	T [K]	Component	Interpretation	CS [mm/s]	QS [mm/s]	H [T]	$\delta_{\text{D/H}}$ [T]	rel. Area [%]	χ^2
KS	77	Fe(III) doublet	Ferrihydrite, lepidocrocite	0.51	0.87		0.6	72.4	0.93
		Fe(II) doublet1	Vivianite	1.30	2.64		0.5	7.9	
		Fe(II) doublet2	Vivianite	1.36	3.10		0.5	6.7	
		Fe(III) sextet1	Goethite	0.53	-0.15	45.6	4.3	13.1	
	30	Fe(III) doublet	Ferrihydrite	0.51	0.81		0.3	53.8	1.15
		Fe(II) doublet1	Vivianite	1.30	2.60		0.5	3.7	
		Fe(II) doublet2	Vivianite	1.25	3.10		0.3	6.1	
		Fe(III) sextet1	Goethite	0.47	-0.15	46.5	4.4	17.6	
		Fe(III) collapse	Poorly crystalline Fe(III) (oxyhydr)oxide, similar to ferrihydrite, with bound organic matter	0.61	0.01	27.5	12.5	18.8	
TIE-1	77	Fe(III) doublet	Ferrihydrite, lepidocrocite	0.48	0.83		0.4	17.3	0.92
		Fe(III) sextet1	Goethite	0.49	-0.12	47.1	1.5	17.2	
		Fe(III) sextet2	Lepidocrocite	0.50	-0.06	42.6	4.0	18.8	
		Fe(III) collapse	Poorly crystalline Fe(III) (oxyhydr)oxide, similar to lepidocrocite	0.46	-0.05	26.8	16.1	46.7	
	30	Fe(III) sextet1	Goethite	0.51	-0.11	48.7	1.2	28.5	1.36
		Fe(III) sextet2	Lepidocrocite	0.62	-0.05	42.9	4.6	21.8	
		Fe(III) sextet3	Lepidocrocite	0.40	0.00	46.1	2.8	22.4	
		Fe(III) collapse	Poorly crystalline Fe(III) (oxyhydr)oxide, similar to ferrihydrite, with bound organic matter	0.43	0.06	31.6	11.9	27.2	
BoFeN1	77	Fe(III) doublet	Ferrihydrite, lepidocrocite	0.47	0.85		0.4	34.6	0.81
		Fe(III) sextet1	Goethite	0.48	-0.13	47.0	1.6	18.3	
		Fe(III) sextet2	Lepidocrocite	0.49	-0.02	42.5	5.9	25.4	
		Fe(III) collapse	Poorly crystalline Fe(III) (oxyhydr)oxide, similar to lepidocrocite	0.51	0.01	14.4	8.1	21.7	
	30	Fe(III) sextet1	Goethite	0.50	-0.12	48.9	1.3	24.6	1.56
		Fe(III) sextet2	Lepidocrocite	0.61	-0.05	42.7	5.1	20.5	
		Fe(III) sextet3	Lepidocrocite	0.37	0.01	45.8	3.4	17.0	
		Fe(III) collapse	Poorly crystalline Fe(III) (oxyhydr)oxide, similar to ferrihydrite, with bound organic matter	0.40	0.01	28.2	13.3	37.9	

Text S4: Mineralogy of BIOS

Culture KS displayed the most complex BIOS composition. More than 50% of the minerals are ferrihydrite with no lepidocrocite present, and it also contains vivianite forming when Fe(II) was added initially to the medium, due to the high phosphate concentration available (4 mM). Detections of vivianite and ferrihydrite aligned with previous studies (Bayer et al., 2023; Nordhoff et al., 2017).

BIOS of TIE-1 and BoFeN1 showed similar mineralogical profiles. In both cases, poorly crystalline Fe(III) oxyhydroxides are detected as expected from biogenic oxidation (Han et al., 2020; Jiao et al., 2005) with goethite and lepidocrocite as secondary minerals (Hansel et al., 2005). In the case of goethite, its formation is favored by the acidic environment resulting from the H^+ generated by the Fe(II) oxidation and the presence of carbonate (Larese-Casanova et al., 2010). Lepidocrocite can also rapidly form from poorly crystalline Fe(III) oxyhydroxides and further transform to goethite, depending on the aging time (H. Liu et al., 2007; Schwertmann & Taylor, 1972; Yan et al., 2015).

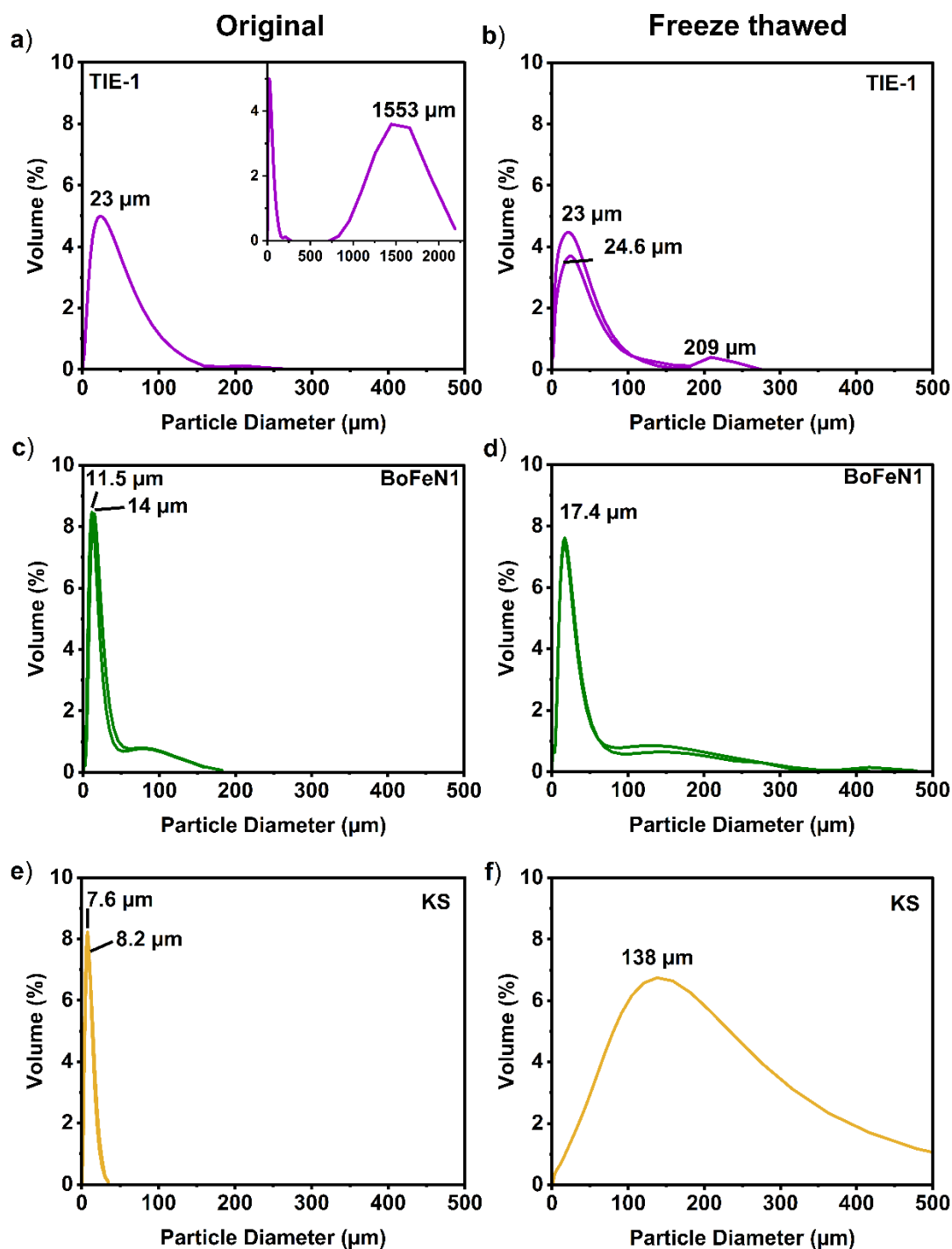


Figure S4. Diameter (μm) of the particulates for each BIOS as determined by a particle size analyzer (Mastersizer 2000), before (left panels) and after freeze-thawing (right panels). Each line within a figure represents a replicate ($n = 2$) for most samples, $n = 1$ for TIE-1 before freeze-thawing and KS after freeze-thawing). The size corresponding to the peak volume % is specified at the top of each line. The inset in (a) is the complete distribution in TIE-1 original BIOS showing the second group of particulates with mean size at 1553 μm .

Table S2. Reduction extent (%) of 6 mM Fe(III) of the BIOS from BoFeN1, TIE-1 and KS in all setups ($n = 3$ each): with original BIOS and freeze-thawed BIOS in the presence and absence of additional carbon, acetate or lactate for *G. sulfurreducens* and *S. oneidensis* MR-1, respectively. Grey values are the lower values when comparing original vs freeze-thawed BIOS.

	<i>G. sulfurreducens</i>				<i>S. oneidensis</i> MR-1			
	No OC added		Acetate		No OC added		Lactate	
	Freeze-		Freeze-		Freeze-		Freeze-	
	Original	thawed	Original	thawed	Original	thawed	Original	thawed
BoFeN1	52±0.02	40±0.01	100±0.01	100±0.01	43±0.05	25±0.02	100±0.02	100±0.19
TIE-1	44±0.01	40±0.08	100±0.01	100±0.01	34±0.01	25±0.02	100±0.02	48±0.08
KS	36±0.01	49±0.02	100±0.02	100±0.03	31±0.00	43±0.01	100±0.01	100±0.05

Table S3. Reduction rate (mM/day) of 6 mM Fe(III) of the BIOS from BoFeN1, TIE-1 and KS in all setups ($n = 3$ each): with original BIOS and freeze-thawed BIOS, in the presence and absence of additional carbon, acetate or lactate for *G. sulfurreducens* and *S. oneidensis* MR-1, respectively. Grey values indicate the lowest values when comparing original vs freeze-thawed BIOS.

	<i>G. sulfurreducens</i>				<i>S. oneidensis</i> MR-1			
	No OC added		Acetate		No OC added		Lactate	
	Freeze-		Freeze-		Freeze-		Freeze-	
	Original	thawed	Original	thawed	Original	thawed	Original	thawed
BoFeN1	0.41±0.05	0.06±0.01	0.72±0.14	0.97±0.18	0.05±0.02	0.04±0.02	0.12±0.04	0.04±0.01
TIE-1	0.16±0.05	0.09±0.02	0.69±0.08	0.56±0.08	0.08±0.02	0.03±0.01	0.13±0.01	0.03±0.01
KS	0.23±0.03	0.29±0.06	0.44±0.10	0.04±0.02	0.09±0.01	0.24±0.06	0.15±0.00	0.10±0.01
Ferrihydrite	-	-	0.07±0.01	0.02±0.00	-	-	0.08±0.00	0.01±0.00

Table S4. Samples used as input for the RDA in Fig. S5.

Predictor variables					Response variables		Compared to original	
#	BIOS source	FeRB	OC addition	BIOS pre-treatment	Reduction rate (mM/day)	Reduction extent (%)	Rate	Extent
1	BoFeN1	<i>G. sulfurreducens</i>	No	Original	0.41	52	Lower	Lower
5	BoFeN1	<i>G. sulfurreducens</i>	No	Freeze-thawed	0.06	40		
2	BoFeN1	<i>S. oneidensis</i>	No	Original	0.05	43	Same	Lower
6	BoFeN1	<i>S. oneidensis</i>	No	Freeze-thawed	0.04	25		
9	TIE-1	<i>G. sulfurreducens</i>	No	Original	0.16	44	Lower	Lower
13	TIE-1	<i>G. sulfurreducens</i>	No	Freeze-thawed	0.09	40		
10	TIE-1	<i>S. oneidensis</i>	No	Original	0.08	34	Lower	Lower
14	TIE-1	<i>S. oneidensis</i>	No	Freeze-thawed	0.03	25		
17	KS	<i>G. sulfurreducens</i>	No	Original	0.23	36	Higher	Higher
21	KS	<i>G. sulfurreducens</i>	No	Freeze-thawed	0.29	49		
18	KS	<i>S. oneidensis</i>	No	Original	0.09	31	Higher	Higher
22	KS	<i>S. oneidensis</i>	No	Freeze-thawed	0.24	43		
3	BoFeN1	<i>G. sulfurreducens</i>	Yes	Original	0.72	100	Higher	Same
7	BoFeN1	<i>G. sulfurreducens</i>	Yes	Freeze-thawed	0.97	100		
4	BoFeN1	<i>S. oneidensis</i>	Yes	Original	0.12	100	Lower	Same
8	BoFeN1	<i>S. oneidensis</i>	Yes	Freeze-thawed	0.04	100		
11	TIE-1	<i>G. sulfurreducens</i>	Yes	Original	0.69	100	Lower	Same
15	TIE-1	<i>G. sulfurreducens</i>	Yes	Freeze-thawed	0.56	100		
12	TIE-1	<i>S. oneidensis</i>	Yes	Original	0.13	100	Lower	Lower
16	TIE-1	<i>S. oneidensis</i>	Yes	Freeze-thawed	0.03	48		
19	KS	<i>G. sulfurreducens</i>	Yes	Original	0.44	100	Lower	Same
23	KS	<i>G. sulfurreducens</i>	Yes	Freeze-thawed	0.04	100		
20	KS	<i>S. oneidensis</i>	Yes	Original	0.15	100	Lower	Same
24	KS	<i>S. oneidensis</i>	Yes	Freeze-thawed	0.1	100		

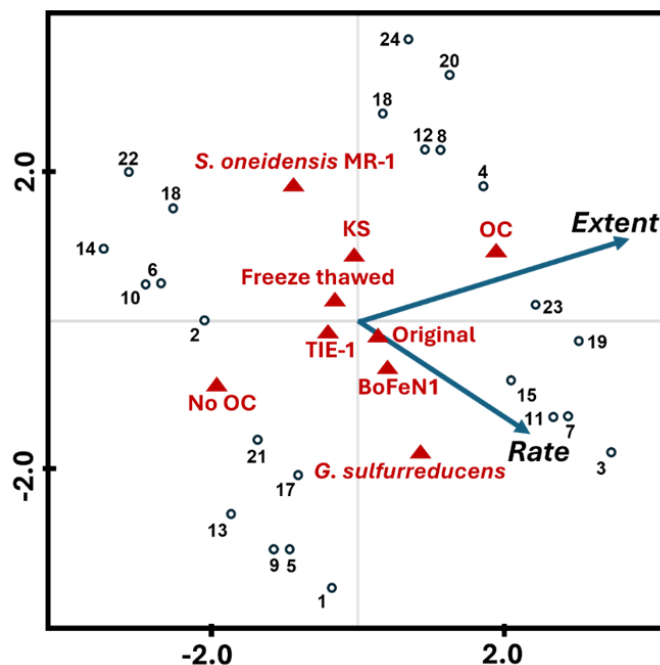


Figure S5. Redundancy analysis testing the relationship between the source of BIOS (i.e. the different FeOB), FeRB, usage of additional carbon and freeze-thawing, on the reduction rate and extent. Blue arrows represent the response variables (reduction rate and extent). Red triangles represent the major predictor variables and circles are the individual setups as defined in Table S4. The analysis indicates that the reduction extent highly depends on the presence of a readily available carbon source, while the rate relies in multiple factors. The FeRB was the main factor affecting the rate, with *G. sulfurreducens* being faster than *S. oneidensis*. Original BIOS also showed a higher rate than those freeze-thawed (frozen).

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Chapter 4. Authors contribution

Cell encrustation leads to cell preservation by entombment after microbial transformation

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This study was conceptualized by M. Mansor. M. Mansor and myself obtained funding for measurements at Diamond Light Source. M. Mansor, C. Escudero and I designed the experiments. I performed the growth experiments and measurements related, except XRD, which was measured by Tsz (Alex) Ho Chiu, C. Escudero performed the hybridizations and M. Wilsch-Bräuninger obtained the SEM images. M. Mansor, C. Escudero and I obtained the cryo-SIM SXT data. M. Mansor, C. Escudero, M. Wilsch-Bräuninger and I analyzed the data. I wrote the original draft of the manuscript and all authors contributed to the review and editing of the final manuscript.

Chapter 4. Cell encrustation leads to cell preservation by entombment after microbial transformation

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Abstract

Iron (Fe) utilizing bacteria have long played an important role in shaping the Earth's biogeosphere. As a by-product of Fe(II) oxidation, Fe(II)-oxidizing bacteria produce Fe(III) nanominerals that in some cases encrust the cells. Complete encrustation leads to cell death while preserving cellular shape and content, with a high potential for biosignature preservation. However, these Fe(III) nanominerals can be transformed by biogeochemical processes, ultimately erasing evidence of its biogenic origin. Here we show that microbial reduction of encrusted cells transforms the Fe(III) nanominerals into larger (20-40 μm) crystalline Fe(II) phosphate mineral vivianite. High resolution 3D imaging showed that encrusted cells act as nucleation sites, becoming entombed within vivianite in the process. This highlights how mineral and microfossil biosignatures are merely transformed – but not destroyed – during microbially driven diagenetic processes, preserving the original Fe(II)-oxidizing bacteria. Our findings propose a new pathway by which microbial iron cycling can produce robust mineral biosignatures, with implications for interpreting ancient iron formations on early Earth and for guiding the search for life elsewhere in iron-rich environments, given the recent suggestion of vivianite deposits in Jezero crater on Mars.¹

Introduction

The reconstruction of the history of life on Earth and the search for life elsewhere highly depends on the evidence of their existence that they leave behind (biosignatures) and our capacity to detect them and determine their biogenicity, rejecting nonbiological processes.² In this study, we focus on biominerals, an example of morphological biosignatures.³ Biominerals are minerals whose formation is controlled or influenced by living organisms, which attributes them particular composition and crystallography.^{4,5} Multiple evidences (e.g., morphology, composition) are necessary to infer biogenicity, because abiotic reactions can form biogenic-like structures – so called biomorphs – that can lead to misidentification of biosignatures.^{6–8} In addition to potential biosignatures themselves, biominerals contribute to the preservation of the related organic matter (cells, cell content and/or extracellular polymeric substances). They prevent or limit degradation by diagenetic processes^{3,9–11} acting as a physical protection via adsorption into mineral surfaces, co-precipitation or entombment.^{9,12–15} The preservation of organic matter within the biomineral matrix leads to stronger and more reliable biosignatures, as organic molecules directly linked to metabolism (e.g., proteins, lipids, amino acids) become incorporated in the minerals.^{14,16} Trapped, the organic molecules are protected against diagenesis and metamorphism, including reactions involving microbes, oxygen and other oxidants or reductants.

Biominerals, such as calcium carbonate (e.g., shells),¹⁷ calcium phosphate (e.g., bones)¹⁸ and Fe(III) (oxyhydr)oxides,¹² are known to help preserve organic matter in the rock record.^{19–21} Fe(III) (oxyhydr)oxides are typically formed via the oxidation of Fe(II) by chemical oxidants (e.g., oxygen, nitrite) or photooxidation; they can also be formed by microorganisms at higher rates than abiotic reactions.^{22,23} Fe(II)-oxidizing bacteria (FeOB) form Fe(III) oxyhydroxides as a by-product of their metabolism.^{24,25} In general, these biogenic Fe(III) oxyhydroxides (BIOS) are potential biosignatures, as some have unique morphology and composition. Mainly due to their associated organic matter, they exhibit higher resistance to transformation into crystalline forms, as well as higher surface area and higher adsorptive capacity than their abiotic counterparts.^{24,26–31}

In the particular case of nitrate-reducing FeOB (NRFeOB), such as *Acidovorax* sp. strain BoFeN1, the newly formed Fe(III) oxyhydroxides precipitate on and within the cell membrane, encrusting the cells.^{32,33} Although this encrustation causes a decrease in nutrient transport and reproductive capacity that probably leads to cell death,^{34,35} it allows the preservation of organic macromolecules and, in some cases, preservation of the unique biomineral morphology, even under diagenetic conditions (250°C/140 MPa).^{32,36}

One of the risk factors for degradation or alteration of the BIOS biosignature is biogenic Fe(III) reduction by Fe(III)-reducing bacteria (FeRB), because BIOS are more bioavailable than abiotically-formed minerals.^{37–39} As a result of the biogenic reduction, FeRB transform BIOS into Fe(II)-bearing minerals or dissolved Fe²⁺.^{40–42} With the aim of investigating BIOS biosignature preservation, here we studied the extent of microbial degradation of BIOS formed by three FeOB with different encrustation degree (**Figure S1**), using two FeRB with different primary reduction mechanisms (direct cell-mineral contact and electron shuttles).

Initial observations of products from microbial Fe(III) reduction of BIOS

We used the BIOS of three different FeOB with increasing degree of encrustation: *Acidovorax* sp. strain BoFeN1 > culture KS > *Rhodopseudomonas palustris* TIE-1 (**Figure S1**). Their biominerals were exposed to reduction by two FeRB with different primary mechanisms of reduction: *Geobacter sulfurreducens* PGA (direct cell-mineral contact) and *Shewanella oneidensis* MR-1 (reduction via electron shuttles). Both FeRB reduced 100% of the BIOS-associated Fe(III) [BIOS_{Fe(III)}] in all cases, leading to the formation of the Fe(II)-phosphate mineral vivianite (Fe₃(PO₄)₂ · 8H₂O), as revealed by micro-x-ray diffraction (μ-XRD) (**Figure S2**). Phosphate was derived from salts dissolved in the cultivation medium and possibly from intracellular phosphate. Fourier transform infrared (FTIR) analysis confirmed vivianite formation (**Figure S3**) that obscured any evidence of organic bonds, highlighting the poor detection of organics by bulk analyses due to their low concentration. Because of the mineral transformation and low OC concentration, the original cell-mineral association in the BIOS, and thus the biosignature, was presumed to be lost.

Morphological analysis of the resulting minerals at the end of the incubation, via scanning electron microscopy (SEM), showed elongated rhomboid-shaped vivianite crystals after reduction of BoFeN1 BIOS_{Fe(III)} by both *G. sulfurreducens* and *S. oneidensis* (**Figure 1**). Minerals formed by *G. sulfurreducens* were thin platy rhomboid crystals with smooth surfaces and edges, 12.1 ± 4.0 μm (*n*=13) long. The crystals stacked together in rosette-like intergrown aggregates. The crystals formed by *S. oneidensis* were prismatic, 9.5 ± 3.8 μm (*n*=13) long and thicker than those of *G. sulfurreducens*. Their rough surfaces contained visible striations and some stacked in stellate aggregates with bladed structures. The striations on the surface were reflected in the higher SSA of the minerals (~4 m²/g), which was higher than that of *G. sulfurreducens* (~2 m²/g) (**Table S1**). Mineral aggregates were seen in some cases loosely associated with the vivianite plates or as

aggregates in the medium, interpreted as residuals from the original Fe(III) (oxyhydr)oxides, mainly botryoidal shaped consistent with ferrihydrite ((Fe^{III})₄₋₅(OH,O)₁₂).

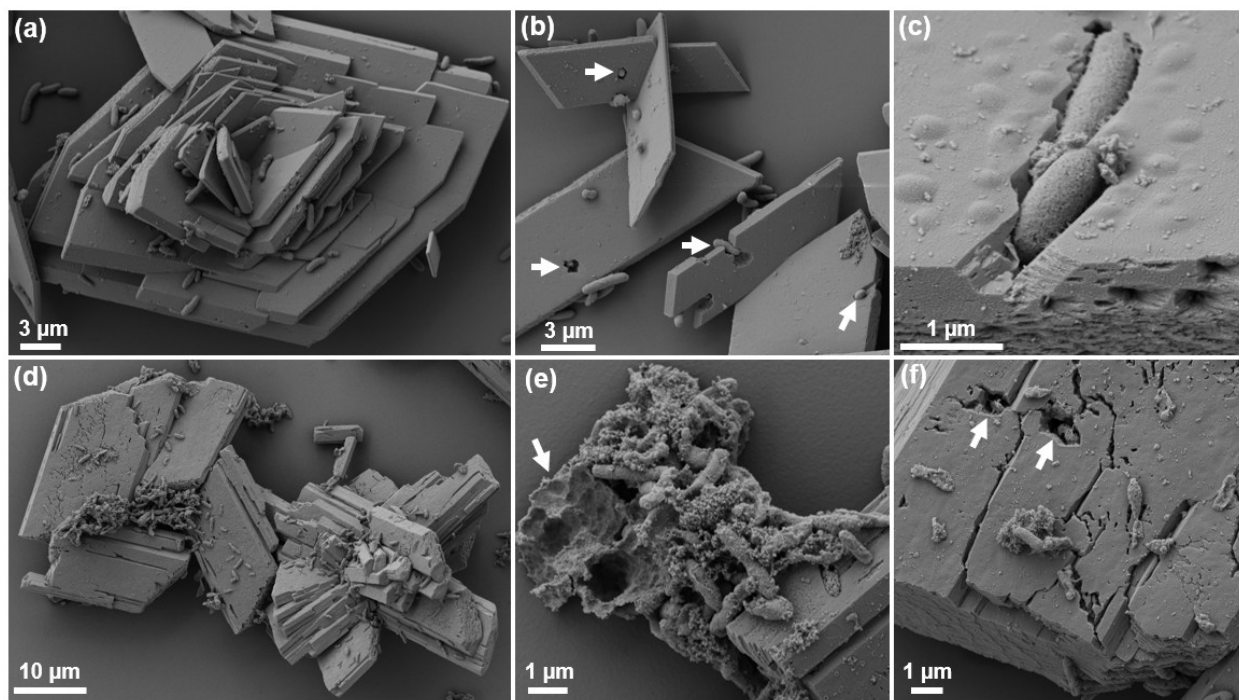


Figure 1. SEM micrographs of the products formed after reduction of BIOS from encrusted cells of BoFeN1. Typical morphology of the vivianite formed in the presence of a) *G. sulfurreducens* were rhomboid-shaped minerals with (b) thin and smooth crystals with cells inside some minerals (white arrows). (c) Closer look into two cells within the crystalline gaps. Blobs extending upward from the mineral surface are artifacts due to beam radiation. In the presence of *S. oneidensis* (d) the vivianite crystals aggregate into a stellate rosette structure. The crystal surfaces were rough and (e) most of the cells were found in groups outside the minerals, in some cases leaving a crust behind (white arrow). Although less common compared to *G. sulfurreducens*, (f) some cells were also found within crystalline gaps (with arrow).

Interestingly, in incubations with BIOS of BoFeN1, partial entombment of cells within the vivianite minerals were observed following reduction by both *G. sulfurreducens* and *S. oneidensis*. Cells were frequently found trapped in gaps in the minerals. Therefore, we followed the progression of this process at different Fe(III) reduction extents (**Figure S4**). With *G. sulfurreducens*, Fe(III) reduction was faster, completed within 10 days. Formation of vivianite was observable early, on day 2 (<10% Fe(II)/Fe(total)). With *S. oneidensis* vivianite formation was observable from day 12 (33% Fe(II)/Fe(total)). The minerals had rougher surfaces with more striations. We interpret the

entombed cells to be BoFeN1, with slightly rough surface structures, due to their smooth surface, different from the wrinkle-like surface of *G. sulfurreducens* or flattened appearance of *S. oneidensis* (**Figure S5**). Some BoFeN1 cells still had remaining Fe(III) coating, while in others, the Fe(III) coating was lost, leaving behind pockmarked cells with porous surfaces.

Compared to *G. sulfurreducens*, there were less observations of cells partially entombed within vivianite, with more cells evident on the mineral surfaces by *S. oneidensis*. We attribute this difference to the different primary reduction mechanisms. Reduction by direct contact, used by *G. sulfurreducens*, creates a localized high concentration of Fe^{2+} around the cells of BoFeN1. The Fe^{2+} that accumulates reacts with the dissolved species, preferentially with the phosphate in the media, which come from the added salts or residual from the cells. The negatively charged functional groups in the cell wall enhance the attraction of the newly formed minerals, promoting their precipitation in the cell wall.

In the contrary, the use of electron shuttles by *S. oneidensis* let the reduction occur beyond the immediate cell surface. Electron shuttles distribute electrons in a larger area in the surrounding medium after Fe(III) reduction, transferring electrons away from the cell and promoting a more dispersed mineral precipitation with less likelihood of cell entombment. Then, although cells can still nucleate the mineral precipitation, the likelihood of mineral precipitation away from the cells stays similar. Additionally, the faster reduction by *G. sulfurreducens* is also in agreement with a more rapid and localized mineral precipitation, while the more arbitrary Fe(III) reduction by electron shuttles occurred less rapidly.

In contrast to BoFeN1, reduction of KS and TIE-1 resulted in no clear evidence of cell entombment. Out of 3 crystals from KS BIOS analyzed and 5 from TIE-1 BIOS, none had entombed cells. Reduction of KS BIOS led primarily to hexagonal flat-plate vivianite crystals with sharp edges and smooth surfaces that in some cases intersected each other (**Figure S6**). Cells were typically observed on top of the vivianite plates but not within, and no clear indication of entombment was seen. The vivianite crystals formed after the reduction of TIE-1 BIOS were rhomboid and flat, thinner and with sharper edges (**Figure S7**). The crystals stacked in random angular orientations indicating multiple nucleation points. No indication of cell entombment was observed. However, in the presence of *G. sulfurreducens*, some vivianite crystals had holes that may indicate nucleation from encrusted cells.

In summary, partial cell entombment was observed when starting with BIOS of BoFeN1, irrespective of the FeRB catalyzing the Fe(III) reduction. In contrast, little to no entombment was observed when starting with BIOS of KS and TIE-1, highlighting the importance of initial encrustation by Fe(III) (oxyhydr)oxides for eventual cell entombment in vivianite.

Confirmation of complete cell entombment

Surface-based SEM imaging showed that cells can be partially entombed (**Figure 1**). To investigate if cells can be completely entombed, which would enhance their preservation potential, we conducted imaging of the interior of vivianite by focused ion beam (FIB) SEM. We focused on BoFeN1 BIOS reduced by *G. sulfurreducens*, considering the frequent observation of partially entombed cells in this sample. **Figure 2** shows representative examples of SEM sequences obtained by FIB milling of vivianite crystals. Because SEM observations were conducted under high vacuum conditions, the risk of artefacts by dehydration and beam damage was high. Specifically, the biogenic vivianite minerals can buckle upon electron beam exposure (e.g. **Figure 1c**) and form cracks within the crystal sheets (e.g. **Figure 2i**). To avoid bias by these artefacts, the entombed cells were analyzed under 3 different conditions: (i) pure minerals (**Figure 2a-g**) and (ii) minerals embedded in epoxy resin (**Figure 2h-l**) for surface protection were imaged at room temperature, and (iii) under cryogenic conditions, vitrified non-dehydrated samples (**Figure 2m-o**), which are closer to their natural state.

Under all 3 conditions, minerals for analysis were preselected by the presence of bacteria on their surface. Entombed cells were detected in most of the minerals formed by *G. sulfurreducens* (17 out of 23 total cells analyzed). Although some cells were only partially entombed (6 out of 17), most bacteria were entombed almost fully by the surrounding mineral (11 out of 17). A small minority of cells were completely entombed by the mineral without any contact to the medium. *S. oneidensis* also showed complete entombment. We hypothesize all elongated cavities of approximately 1 μm length within the vivianite being derived from cells, although the density and structure of the cytoplasm varied (e.g. **Figure 2l**). As mentioned above, we interpreted the entombed cells as BoFeN1 based on their size and surface morphology. However, morphological identification is known to be ambiguous, prompting the use of targeted labelling techniques to more accurately determine the cell identity.

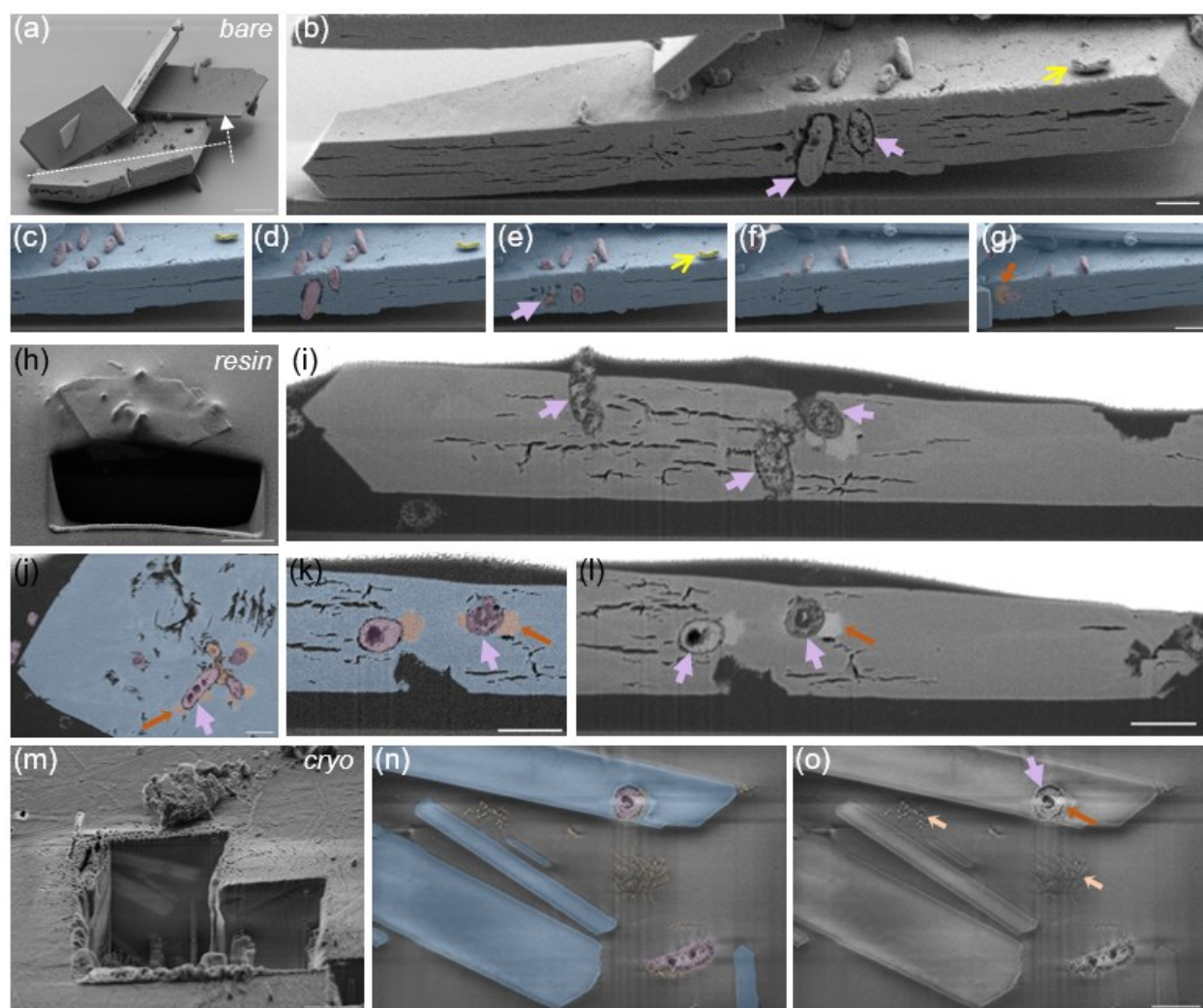


Figure 2. SEM micrographs of a representative FIB-milled vivianite crystal from reduction of BoFeN1 BIOS by *G. sulfurreducens*. Samples in (a-g) were fixed, dehydrated and HMDS-dried at room temperature. (h-l) Were fixed, dehydrated and resin-embedded crystals at room temperature. Those in (m-o) were high-pressure-frozen, imaged under cryogenic conditions. (a, h and m) Overview images showing the planes during FIB-SEM milling. White dashed line and arrow in (a) indicate the direction of milling progress. Scale bars are 5 μm . (b) Dried crystal showing the cross-section (slice 106) of two entombed bacteria (pink arrows) and bacteria attached to the surface of the crystal. The bacterium on the right (yellow open arrow) shows the wrinkled surface typical for *G. sulfurreducens*. (c-g) Segmented views through the same crystal at subsequent milling positions (Slice 88, 98, 108, 118, 128). (i) Resin-embedded crystal showing the cross-section of 3 entombed bacteria (pink arrows) under the resin layer (white, top). (j) Reconstructed view through the crystal plane (parallel to resin-surface and stub-support) showing 4 segmented bacteria (pink) entombed in the vivianite crystal (blue). (k, l) Cross-section views through the same

position of this crystal showing two entombed bacteria. The bacterium on the left is fully entombed, whereas the bacterium on the right is partially exposed, remaining in contact with the surrounding medium (see supplementary video). Note the electron-translucent patches adjacent to the bacterial surface, interpreted as remaining Fe(III) (oxyhydr)oxides (orange segmentation and beaded arrows). (n, o) Segmented and un-segmented view through vivianite crystals imaged under cryogenic conditions with an entombed bacterium. In addition to patches next to the bacterium within the crystal, some globular structures and flake aggregates are visible in the frozen medium (light orange small-beaded arrows). No striations within the vivianite crystals are observed in aqueous samples under cryogenic conditions which implies that they are dehydration artefacts in the room temperature samples (a-l). Scale bars in (b), (g) for (c-f), (i-l), (o) for (n), are 1 μm . Pink arrows point to entombed bacteria (presumably FeOB), yellow open arrows point to presumably FeRB, orange beaded arrows point to Fe(III) (oxyhydr)oxides patches and small light-orange arrows point to mineral aggregates in the culture medium.

Species identification of entombed cells

To unambiguously assign the identity of the entombed cells, we applied a correlative microscopy workflow combining fluorescence in situ hybridization (FISH), structured illumination microscopy and soft X-ray tomography under cryogenic conditions (cryo-SIM-SXT). Fluorescence in situ hybridization allowed the identification of cells by using specific FISH probes for targeting FeRB and the general DNA stain SYTO-9. Thus, Fe(III)-reducing bacteria emit fluorescence from both SYTO-9 and the FISH probe, whereas Fe(II)-oxidizing bacteria emit signal only from SYTO-9, enabling clear discrimination between both groups. Cryo-SIM was subsequently used to localize and spatially resolve the labeled cells within the mineral matrix, providing fluorescence-based spatial context. Finally, SXT was applied to the same regions, generating high-resolution 3D tomograms of the cell–mineral associations, thereby directly linking microbial identity with structural context.

We focused our cryo-SIM-SXT analysis on the products of BoFeN1 $\text{BIOS}_{\text{Fe(III)}}$ reduction by *G. sulfurreducens*, given the complete entombment of cells previously confirmed by FIB-SEM (**Figure 2**). Correlative imaging of identical regions allowed direct visualization and identification of cells in relation to the vivianite crystals (**Figure 3**). Cells embedded within the mineral were assigned to BoFeN1 based on their exclusive SYTO-9 signal, whereas cells located outside the crystal showed both SYTO-9 and FISH signals and were thus identified as *G. sulfurreducens*. Importantly, the positive signals of FISH targeting BoFeN1 in cells located within the vivianite

crystals confirm that probe penetration was not hindered by mineral entombment and ruling out false negatives due to physical shielding. However, this diffusion also reveals the limitations of the vivianite entombment. Molecules that could degrade the cells, such as reactive radicals, could diffuse inside the mineral as did the stain.

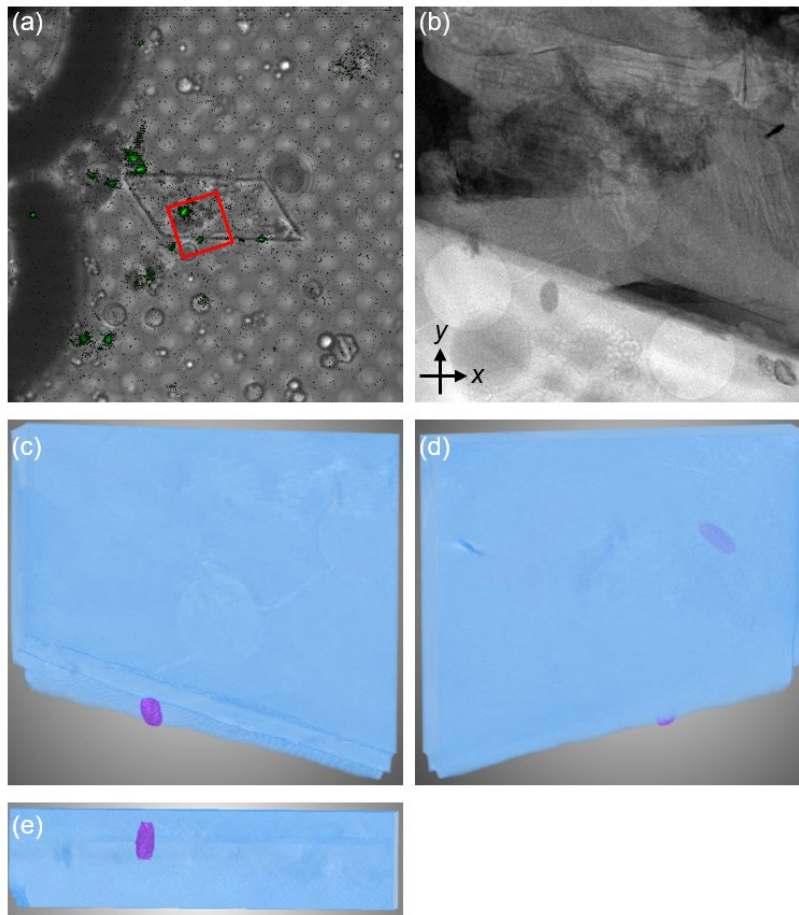


Figure 3. Representative cryo-SIM-SXT analysis of vivianite crystal resulting from BoFeN1 BIOS reduction by *G. sulfurreducens*. (a) Structured illumination microscopy image of the crystal where at least two cells stand out, one on the edge of the crystal and another one that seems in the middle. The green staining (SYTO-9) indicates both cells are BoFeN1. *G. sulfurreducens* cells, labelled with both FISH and DNA staining, would appear in red. (b) Soft x-ray tomograph of the area within the red square in (a). False color of the tomograph shows that (c) the cell in the middle is not on top of the mineral in this phase on the vivianite. (d) A 180° rotation on the y axis and 30% of opacity lets the cell visible in the interior, (e) while a 90° rotation in the x axis shows that the cell is inside the vivianite and not on its surface.

Initially encrusted cells remained preserved despite the microbial reduction of their original Fe(III) (oxyhydr)oxide coating. FeOB encrustation acted as a nucleation site for the formation of a larger secondary mineral phase, vivianite, thereby enhancing the preservation potential of the originally encrusted cell. Moreover, the presence of darker zones within the vivianite crystals (**Figure 3b**) corresponding to regions of higher carbon signal in the SXT analysis, supports the possible presence of organic-rich domains associated with the mineral phase. Although we cannot rule out that this carbon could originate from sample preparation (e.g., DNA stain or formaldehyde fixation), it is unlikely, as no corresponding signal is detected in the SIM data (**Figure 3a**).

Implication to biosignature preservation

Fe(III) (oxyhydr)oxides, including BIOS and microbial fossils, have been interpreted as biosignatures.^{26,43–46} This interpretation is supported by experimental studies that showed limited morphological changes of BIOS after exposure to high temperatures and pressures representative of late diagenesis.³⁶ Nonetheless, it is well accepted that BIOS are susceptible to early stage microbial Fe(III) reduction, raising doubts as to their long term preservation. Furthermore, the organic content associated with these structures may not always be preserved,⁴⁷ unless the organics are entombed within minerals.^{32,48,49}

Here, we showed that after BIOS_{Fe(III)} reduction, the encrusted cells of BoFeN1 served as nucleation sites for the formation of vivianite (**Figures 2-3**), indicating that despite mineral transformation, entire cells and/or related organics can still be preserved by entombment. Cells of nitrate-reducing *Tessarococcus lapidicaptus*, for instance have shown to act as nucleation sites for vivianite nanocrystals, although cell entombment was not observed.⁵⁰ The large size of the vivianite (17-28 µm long and 0.4-2 µm wide) is a prerequisite for entombment, as smaller minerals may only partially enclose the cells without offering protection against degradation. Thus, large vivianite minerals can act as hosts for biosignatures of FeOB. It is worth noting that the FeOB must be Fe-encrusted as we did not find entombment to happen with KS or TIE-1 BIOS, those with less encrustation degree. This would bias the preservation towards FeOB that were initially encrusted with Fe(III) nanominerals, giving insights into the environmental conditions, because encrustation occurs preferentially at high Fe(II) concentration and low Fe minerals concentration, although potentially leading to bias in diversity and ecology of FeOB.^{34,51,52}

The relevance of this biosignature preservation pathway in natural environments is worth discussing. First, FeOB encrustation is usually observed in the laboratory at high Fe(II) concentrations

(e.g., mM levels) that are not common in the environment. The occurrences of cell encrustation by Fe(III) nanominerals in nature are rarely documented.^{53–55} Microaerophilic Fe(II) oxidizing bacteria can generate encrusted structures such as twisted stalks, tubular sheaths and dreadlocks, while the cells themselves escape encrustation.^{30,56} We speculate that these unique structures may be entombed by vivianite following microbial Fe(III) reduction.

Second, vivianite is known to be a thermodynamically stable mineral under anoxic, reducing conditions, acting as a sink for phosphorous in sediments typically from the quaternary to recent times (Holocene-Pleistocene).⁵⁷ Although geochemical models of Precambrian oceans (>2.5 Ga) suggest the formation of vivianite-like Fe(II) minerals, when seawater was rich in dissolved Fe²⁺, reports of vivianite in ancient rocks are rare.⁵⁸ The oldest vivianite deposits are found in formations typically dated to early Holocene <8000 years BP).⁵⁹ In modern environments, they are typically found in lacustrine Fe-rich environments. Interestingly though, vivianite has been recently detected as part of a mineral assemblage on Jezero crater on Mars.¹ This opens the exciting possibility of finding evidence of life on Mars. It further highlights the value of sample return mission for imaging the interior of those minerals – to search for evidence of cellular structures – with techniques that are more versatile than those able to be carried on board Martian rovers.

Compared to other minerals that are known to entomb microfossils, quartz (SiO₂) and calcite (CaCO₃) are especially recognized for their role in the preservation of morphological biosignatures.^{60,61} Cells are trapped during quartz precipitation, a common process in silica sinters and hydrothermal deposits, where some of the oldest evidence of life has been found (ca. 3.5 Ga).^{62,63} Precipitation of carbonate also traps cells and preserves microbial structures mainly in marine environments, often in the form of stromatolites (ca. 3.4-3.5 Ga).^{62,64} Compared to quartz and calcite, vivianite often displays smaller particle sizes (μm vs mm-cm) and higher specific surface areas (1-4 m²/g vs 0.01-2 m²/g).^{65–67} These indicate higher dissolution potential of vivianite compared to quartz and calcite. Furthermore, vivianite is expected under Fe-rich conditions while quartz and calcite have a more cosmopolitan distribution.^{68,69} Hence, cells entombed within vivianite are potential biosignatures only in specific niches, such as lacustrine and marine sediments on Earth, or even in Fe-rich Martian deposits.

Conclusion

Biomineralization is a promising way of preserving biosignatures. First, biominerals often have unique morphologies^{30,70,71} that are usually associated with organic molecules, including lipids

and extracellular polymeric substances.^{72–75} Second, biominerals are resistant to long-term diagenesis (high temperatures and pressures),^{36,48,76} allowing their preservation in the geological record. However, early diagenesis, driven by microorganisms, is also a critical factor that threatens the preservation of biominerals. Here we found that the mineral part of BIOS was transformed into vivianite by FeRB, losing the original mineral signature, while preserving the organic part as entire cells entombed inside the newly formed minerals. This entombment correlated with the initial degree of encrustation of the FeOB cells, consistent with the notion that encrustation provides protection for organic matter leading to its preservation, even against microbial degradation. Encrusted FeOB cells acted as nucleation sites, leading to their preservation and providing another mineral where microbial biosignatures can be expected and looked for mainly in lacustrine and Fe-rich environments.

Methods

BIOS sources

Three FeOB were used for producing the BIOS based on three different Fe(II) oxidizing metabolism: *Rhodopseudomonas palustris* TIE-1 (photoferrotroph), enrichment culture KS (autotrophic NRFeOB) and *Acidovorax* sp. BoFeN1 (mixotrophic NRFeOB). FeOB were cultivated in 22 mM bicarbonate-buffered mineral medium at pH 7 (Ehrenreich & Widdel, 1994) under anoxic conditions with a headspace of N₂/CO₂ (90:10 v/v %) in the dark at 28°C for BoFeN1 and KS, and at 20°C and illuminated for TIE-1. BoFeN1 medium contained 10 mM Fe(II) (as Fe₂Cl), 6 mM NO₃⁻ (as NaNO₃) and 2 mM acetate (as CH₃COONa). Because the BoFeN1 medium had lower phosphate concentration (1 mM), 3.5 mM NaCl were added to maintain the ionic strength in the medium. KS medium contained 10 mM Fe(II) (as Fe₂Cl) and 2 mM NO₃⁻ (as NaNO₃). TIE-1 medium contained 3 mM Fe(II). Precultures were grown in the same conditions as described above and the inoculum was 1%, 5% and 3% (vol/vol) for KS, BoFeN1 and TIE-1, respectively. BIOS were collected by centrifugation at the end of oxidation (Fe(II)/Fe(total) ratio 94.4±1.5%, 92±2.0% and 93±0.2% for TIE-1, BoFeN1 and KS, respectively). The supernatant was exchanged twice with FeRB medium. After the third centrifugation all the BIOS were concentrated in respective 200 mL serum bottles.

Cultivation of microorganisms

G. sulfurreducens and *S. oneidensis* were grown in a 30 mM bicarbonate-buffered mineral medium (Ehrenreich & Widdel, 1994) under anoxic conditions with a headspace of N₂/CO₂ (90:10 v/v %) in the dark at 28°C and pH 7. The medium was amended with ~6 mM BIOS_{Fe(total)}, 5 mM of

acetate for *G. sulfurreducens*, 5 mM lactate for *S. oneidensis*, and initial cell concentration of $\sim 10^5$ cells/mL.

Mineral phases

Samples for μ -XRD and FTIR spectroscopy were collected at the end of reduction. Inside of a glovebox, ca. 5 mL of each setup were centrifuged at 12045 g for 5 minutes. The supernatant was removed and the pellet was left to dry inside the glovebox for up to 24 h. The crystalline mineral phases were identified by μ XRD in a Bruker's D8 Discover GADDS XRD2 micro-diffractometer equipped with a standard sealed tube with a Co-anode (CoK α radiation, $\lambda = 0.17903$ nm, 30 kV/30 mA, 30 kV/30 mA). Reflection signals were detected in a range from 10° to 70° 2-theta in 240 s. The resulting diffractograms were analyzed using the software Match! (version 3.6.2.121).

Organic and inorganic bonds within the reduction products were determined by means of FTIR. About 1 mg of dried sample was mixed with 250 mg of KBr and pressed into a pellet. Spectra were collected under vacuum from 370 to 4500 cm^{-1} for an average of 256 scans at an instrument resolution of 4 cm^{-1} using a Bruker Vertex 80v FTIR spectrometer (Bruker, USA). The spectra were smoothed with an FFT filter and the baseline was fitted with the first derivative.

The specific surface area (SSA) of the minerals was determined by adsorption/desorption of N_2 using a Micromeritics Gemini VII surface area and porosity analyzer (Micromeritics Instrument Corporation, USA). N_2 adsorption was quantified from outgassed samples over a 24-hour period at partial pressures ranging from 0.05 to 0.3 P/P_0 and at 77 and 298 K. Particle size in suspension was measured using a particle size analyzer (Mastersizer 2000, Malvern Instruments, United Kingdom). Samples were added directly to the Hydro 2000S dispersion unit one mL at a time until the obscuration was between 10-20% with distilled water as dispersant (total volume of 150 mL). Measurements were conducted four times per sample, on duplicate samples, with three cleaning cycles in between each sample.

Chemical analysis

We imaged BIOS before and after complete reduction by *G. sulfurreducens* and *S. oneidensis*. In the case of BoFeN1 BIOS, we also imaged after 25, 50 and 75% reduction of BIOS_{Fe(III)}. Percentage of reduction was followed by colorimetry using the ferrozine assay.^{77,78} Ferrozine-Fe(II) complex in all samples was measured in triplicate and in the dark at 562 nm in a plate reader

(Multiskan GO; Thermo Fisher Scientific, USA). For quantification of Fe(total), 20 µl of sample were diluted 1:4 in hydroxylamine hydrochloride (10% w/v in 1 M HCl) for 30 min before adding ferrozine reagent at a 1:1 ratio. The ferrozine-Fe(II) complex was measured after 5 min of incubation. For quantification of Fe(II) content, the samples were diluted 1:4 in 1 M HCl and then incubated for 5 min with 1:1 ferrozine agent before measurement.

Scanning electron microscopy

To prepare the samples for SEM, 100 µl of anoxic sample were fixed with glutaraldehyde (25% Electron Microscopy grade) at a ratio of 1:9 for 24 h in the cold. After fixation, the samples were washed by centrifugation and 900 µl of supernatant was exchanged with filter sterilized (0.45 µm pore size) milli-Q water, twice. Then, 50 µL of the fixed and washed samples were added to a 12 mm poly-L-lysine coated cover glass slide inside a 24-well plate. After 30 min settling time, the samples were sequentially dehydrated with 1 mL of 25%, 50%, 75%, 90% and 96% ethanol for 15 min at each step. Coated glass (12 mm) coverslips were prepared by glow discharge for 25 s at 0.39 mbar in a PELCO easiGlow by adding 35 µl of 0.01% w/v solution of poly-L-lysine (Sigma Aldrich) and let to dry for 1h at 50°C and overnight at RT to provide a hydrophilic surface for minerals and cells. A dehydration step with 100% ethanol followed which was repeated twice with 30 min incubation time each. Another transition incubation with 1:1 ratio of 100 % ethanol:HMDS (1,1,1,3,3,3-Hexamethyldisilazan, Roth) was performed for 30 min, before incubating twice with 100% HMDS. The last incubation (250 µl of 100% HDMS) was allowed to evaporate overnight in a fume hood with the well plate lid slightly open. Afterwards, the coverslips were mounted on aluminum stubs with adhesive carbon tape (PLANO, Wetzlar). To prevent charging during analysis, the slides were sputter-coated with a ca. 8 nm platinum layer (sputtering 80 s at 5×10^{-2} mbar, 30 mA, and a working distance of 35 mm) with a BAL-TEC SCD 005 sputter coater.

Imaging at high resolution was performed on a ZEISS Crossbeam 550 L Scanning Electron Microscope with an acceleration voltage of 2 kV and probe current of 100 pA at a working distance of 5 mm. Micrographs were recorded with the SESI (secondary electrons - secondary ions) detector.

FIB-milling in combination with SEM image acquisition on the Zeiss Crossbeam 550L was performed to analyze the cell entombment inside the minerals. Room temperature samples were prepared either (i) as for SEM morphological analysis, *i.e.* dehydrated and dried mineral/bacteria setups on poly-L-Lysine-coated glass coverslips, sputter-coated with an 8 nm conductive platinum

layer, or (ii) with resin embedding. For the latter, after ethanol-dehydration, samples were infiltrated with a 1:1 ratio of 100% ethanol and embedded in Epoxy resin for 15 min twice and exchanged for pure resin. After 1 h of infiltration, almost all resin was drained off and the samples polymerized at 60 degrees for 3 days. The glass coverslips were removed by dipping into liquid nitrogen and the sample mounted on a carbon tab with a minimal amount of Epoxy resin as glue (60 degree polymerization overnight). The samples were sputter-coated with 8 nm platinum prior to SEM imaging. Mineral particles which were approximately parallel to the sample support and showed some attached bacteria were selected as regions of interest in the plain dry-mounted samples and in the resin-embedded samples in regions with thin overlaying resin with a 5kV imaging beam. Cross-sections through the structures of interest (dry mounted or resin-embedded) were roughly milled at 54 degrees stage inclination with a 30 kV:7 nA Ga-FIB beam. The working distance was 5.1 mm. Room temperature samples were milled with a 30 kV:100pA FIB beam (15 nm slice thickness for resin embedded samples, 50 nm slice thickness for dry sample). Serial section imaging of the freshly exposed sample surface was performed with the SESI detector at 1.5 kV accelerating voltage and 20 pA probe current and 7.5 nm xy-pixel resolution (resin sample)/11 nm xy-pixel resolution (dry sample).

To avoid fixation or dehydration and drying artefacts, an additional batch of BoFeN1 BIOS reduced by *G. sulfurreducens* was imaged under aqueous, cryogenic conditions. Cryo-samples were prepared by rapid vitrification by high pressure freezing: a volume of 3 μ L of an unfixed culture was transferred into 3 mm aluminum carriers with 100 μ m depression. An assembly was formed with a fine-grain (5 μ m) sand paper-polished flat carrier lid which was quickly frozen in a pre-cooled and pressurized compact high-pressure freezer (Engineering Office M. Wohlwend GmbH, Switzerland). Then, samples were transferred into liquid nitrogen for storage. In preparation of imaging, a carrier-sandwich was mounted on a cryo-holder under liquid nitrogen in a Leica Vacuum Cryo Manipulation (VCM) loading station, prior to transfer employing a Leica Vacuum Cryo-Transfer unit (VCT) 500 to a Leica EM ACE600 high vacuum coating device (Leica Microsystems, Wetzlar, Germany). The carrier-assembly was opened by *freeze fracture* in the coating device and the freshly exposed sample surface was sputter coated with 6 nm platinum at 2×10^{-2} mbar at -196°C . Afterwards, it was transferred with the VCT500 shuttle to the pre-cooled cryo-stage (-147°C) on the microscope with a Leica VCT500 cooling control. After tilting the sample to 54° and adjusting a working distance of 5.1 mm, a rough trench was milled into the sample with a 30 kV:1.5 nA Ga-FIB beam until an object of interest was reached, then the exposed surface was refined with a 30 kV:700 pA beam. Serial section imaging was performed at 30 kV:300

nA / 3 nA FIB milling (20 nm/14.5 nm slice thickness) and 2 kV, 100 pA / 20 pA imaging with 14.5 nm pixel size (xy) with the SESI detector.

Fluorescence in situ hybridization (FISH)

Samples were fixed with 4% formaldehyde for 2 h at 4°C. Fixation was stopped by washing with phosphate-buffered saline (PBS; NaCl 8 g L⁻¹, KCl 0.2 g L⁻¹, Na₂HPO₄ 1.44 g L⁻¹, KH₂PO₄ 0.24 g L⁻¹), and samples were stored at 4°C until further processing. FISH was performed on BIOS following the protocol described by Glöckner et al. (1996).⁷⁹ Cells of *G. sulfurreducens* were targeted using the Cy3-labeled probe GEO2 (S-S-Gsulf-0207-a-A-19) together with the helper oligonucleotides HGEO2-1 and HGEO2-2⁸⁰ (Biomers, Ulm, Germany), using a hybridization buffer containing 10% formamide. Hybridizations were carried out overnight in 0.5 mL microcentrifuge tubes. Buffer exchanges were performed by gentle centrifugation to pellet BIOS, followed by replacement of the supernatant. Samples were counterstained with SYTO-9 (Thermo Fisher Scientific, USA) following the instructions of the manufacturer. Cells of *G. sulfurreducens* were identified by the combined FISH signal and DNA staining, whereas BoFeN1 cells were operationally defined as those showing only SYTO-9 fluorescence. After staining, samples were kept in sterile milli-Q water and stored at 4°C until further processing.

Cryo-Structured Illumination Fluorescence and Soft X-ray Microscopy (Cryo-SIM-SXT)

Samples were transported from the University of Tübingen to beamline B24 at the Diamond Light Source, UK. About 2 µl of samples were deposited onto transmission electron microscopy grids (Quantifoil R 2/2 on 200 gold mesh), mixed with 2 µL of 250 nm gold nanoparticles fiducials, and plunge-frozen in liquid nitrogen-cooled liquid ethane. Three replicates were prepared for each sample and cryopreserved in liquid nitrogen until imaging.

Correlative cryogenic fluorescence imaging was performed using a cryo-structured illumination microscopy (cryo-SIM) setup (UltraXRM-S/L220c, Carl Zeiss X-ray Microscopy) equipped with a cryo-stage maintained below -80°C. Grids were mapped in mosaic mode under brightfield illumination to identify regions of interest (ROI), followed by fluorescence imaging using excitation lasers at 488 and 561 nm and appropriate emission filter sets. Structured illumination image stacks were acquired at selected positions with z-stack acquisition adapted to sample thickness, enabling high-resolution localization of fluorescently labeled cells within the mineral matrix. Multichannel fluorescence images were reconstructed using structured illumination reconstruction (SoftWoRx), and chromatic aberrations between channels were corrected using Chromagon

software⁸¹ with calibration-based alignment parameters, ensuring accurate spatial overlap of fluorescence signals. At least 4 ROIs were analyzed per sample.

Following cryo-SIM imaging, samples were transferred under cryogenic conditions to the SXT instrument at beamline B24. Samples were maintained in a liquid nitrogen-cooled vacuum chamber (<114 K) throughout data acquisition to preserve cryogenic conditions. Initial grid mapping and region selection were performed using visible light microscopy, and regions of interest identified in cryo-SIM were correlated using grid coordinates.

Tilt series were acquired using a Pixis-XO:1024B CCD camera (Teledyne Princeton Instruments) with X-ray illumination at 500 eV ($\lambda = 2.48$ nm), which provides contrast based on differential absorption of organic-rich (carbon-dense) versus mineral phases within the water window energy range. Tomographic datasets were collected over an angular range of -65° to $+60^\circ$ with 0.5° increments and exposure times of 0.5–1 s per projection, resulting in ~220 projections per dataset. The effective spatial resolution of the SXT setup was ~30–50 nm, with a voxel size of ~10 nm.

Image processing

Image processing for SEM images and cropping of FIB-SEM scanned regions of interest was done with Fiji/ImageJ software⁸². All 3D volumes (**Figure 2**) were rendered in ORS Dragonfly v. 2024.1 3D World (Comet). For cryo stacks, a vertical destriping filter was applied to reduce curtaining effects with a wavelet decomposition (db 2, sigma 6 / db 4 sigma 1), followed by a local contrast enhancement CLAHE in Dragonfly. Registration (SSD, only translation allowed) and linear drift correction were used to align the slices in Dragonfly. Single frames were manually segmented.

For the room temperature FIB-SEM stacks, images were registered using a feature-based SIFT alignment with only translation allowed, either in Dragonfly (resin stack) or the TrakEM2 plugin in Fiji (plain dry-mounted stack)⁸³ followed by linear drift correction in Dragonfly. Deep learning was applied to train a 2.5D U-Net with depth level 5 and 3 slices, to automatically recognize background, vivianite crystals, bacteria and ferrihydrite attachments in Dragonfly using around 5 hand-annotated frames for ground-truth. After an initial training round, 1-2 additional annotated frames were added and training continued. The full volume was then segmented and artifacts in the segmentation were manually corrected. Movies were generated with the segmented 3D rendering.

Image reconstruction of tomograms was done with IMOD⁸⁴ using 30 iterations^{82,85} and gold nanoparticles as fiducial markers. Segmentation and 3D renders were built with Dragonfly (Comet Technologies Canada, Canada). The regions of interest were those with entombed cells. For segmentation, first the intensity values were inverted to improve the signal and the differentiation of the different components. Then, new ROIs were created for each component (minerals or individual cells). The area of each component was selected manually in at least ten slices and then interpolated to all the stacks.

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Supplementary material for: Cell encrustation leads to cell preservation by entombment after microbial transformation

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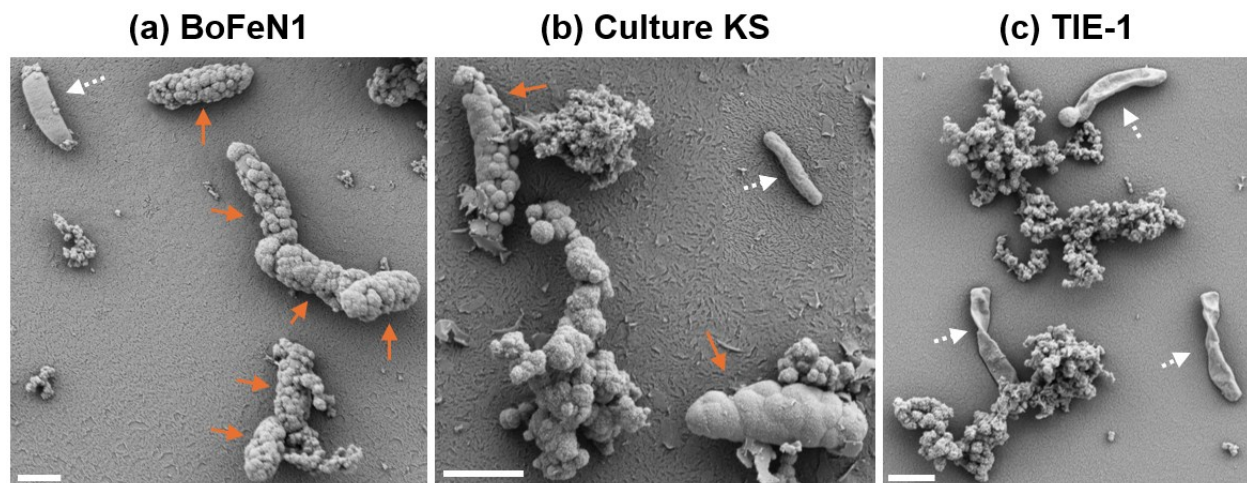


Figure S1. Different encrustation degree among FeOB. a) In *Acidovorax* sp. BoFeN1, the majority of the cells are fully encrusted (full arrows), while some remain mostly unencrusted (dashed arrow). Despite the extracellular polymeric substances (EPS) they secrete to avoid encrustation, the (oxyhydr)oxides they produce fill the periplasm and then the outer membrane.^{1,2} b) Within the enrichment culture KS, *Ferrigenium straubiae*, an autotrophic microaerophilic Fe(II)-oxidizer bacterium (Becker & Kappler, 2025) dominates the enrichment (>98%).⁴ Fe(II) oxidation leads to the encrustation of other cells that form part of the flanking community (full arrows), while *Ferrigenium straubiae* is typically not encrusted (dashed arrow). c) *Rhodopseudomonas palustris* TIE-1 is a photoautotrophic Fe(II)-oxidizer that oxidizes Fe(II) enzymatically at the surface of the cell membrane and do not exhibit encrustation.³ The cells appear flattened due to poor preservation during SEM sample preparation. Scale bar is 1 μm.

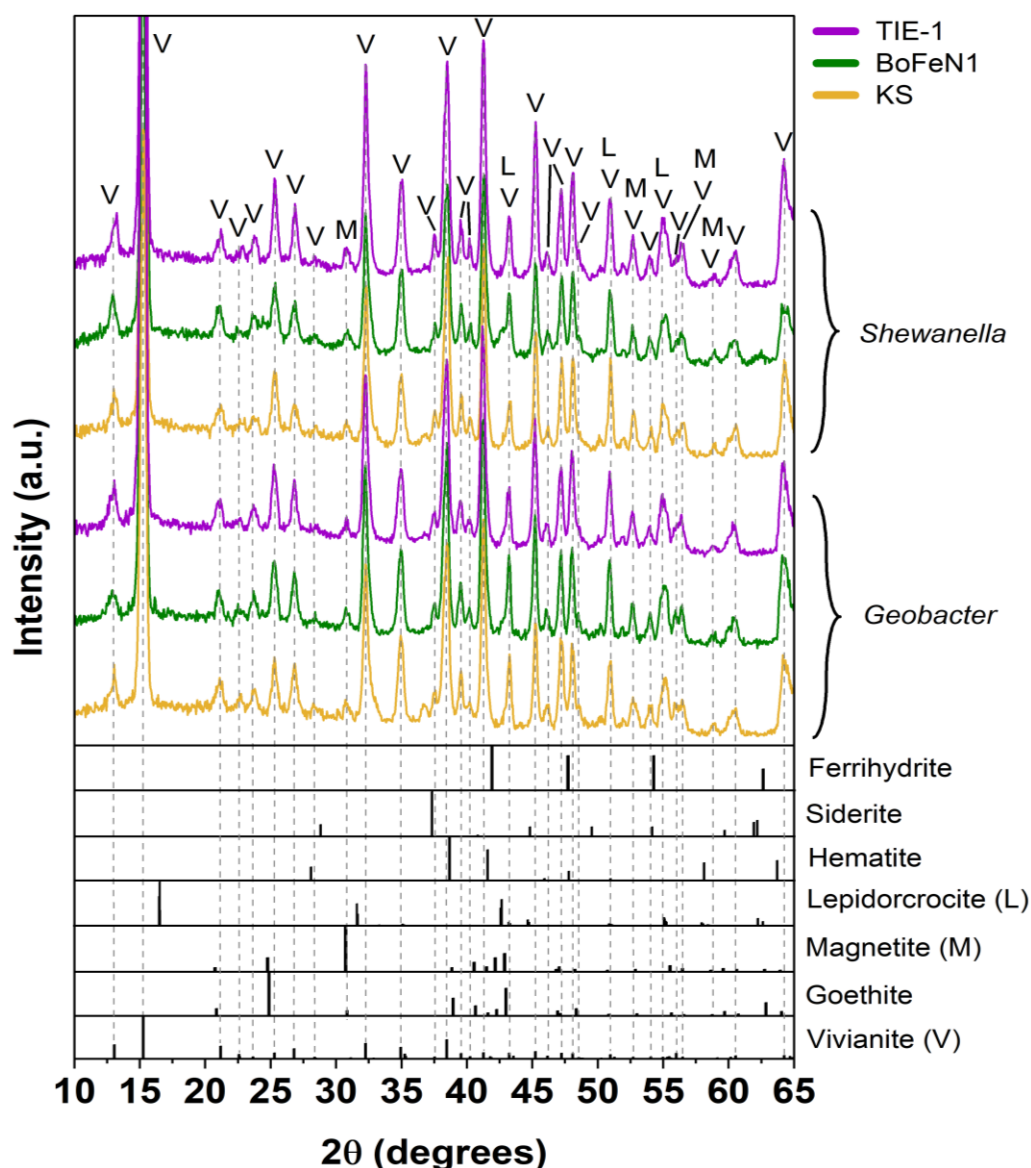


Figure S2. Diffractograms of the products of BIOS_{Fe(III)} reduction from *S. oneidensis* (top three) and *G. sulfurreducens* (the following three). Reference XRD spectra are shown in black at the bottom. Biogenic Fe(III) reduction typically leads to the formation of magnetite, siderite and vivianite. Here, the main mineral product was vivianite in all cases, consistent with the moderate phosphate concentration in the medium (4 mM) of the FeRB.⁵ When phosphate concentration is low, magnetite is expected to be a major product. In this case, it is a minor product, seen in the diffractogram and visually as small black particles (not shown). As expected, siderite was not formed, because its formation is only favored in the absence of phosphate.⁶ Reflections at 43, 51 and 55° 2-theta overlapped between vivianite and lepidocrocite. Even though lepidocrocite was part of the initial BIOS and could remain behind, its three main reflections at 16, 32 and 42.5° 2-theta are missing, suggesting its absence or low concentration.

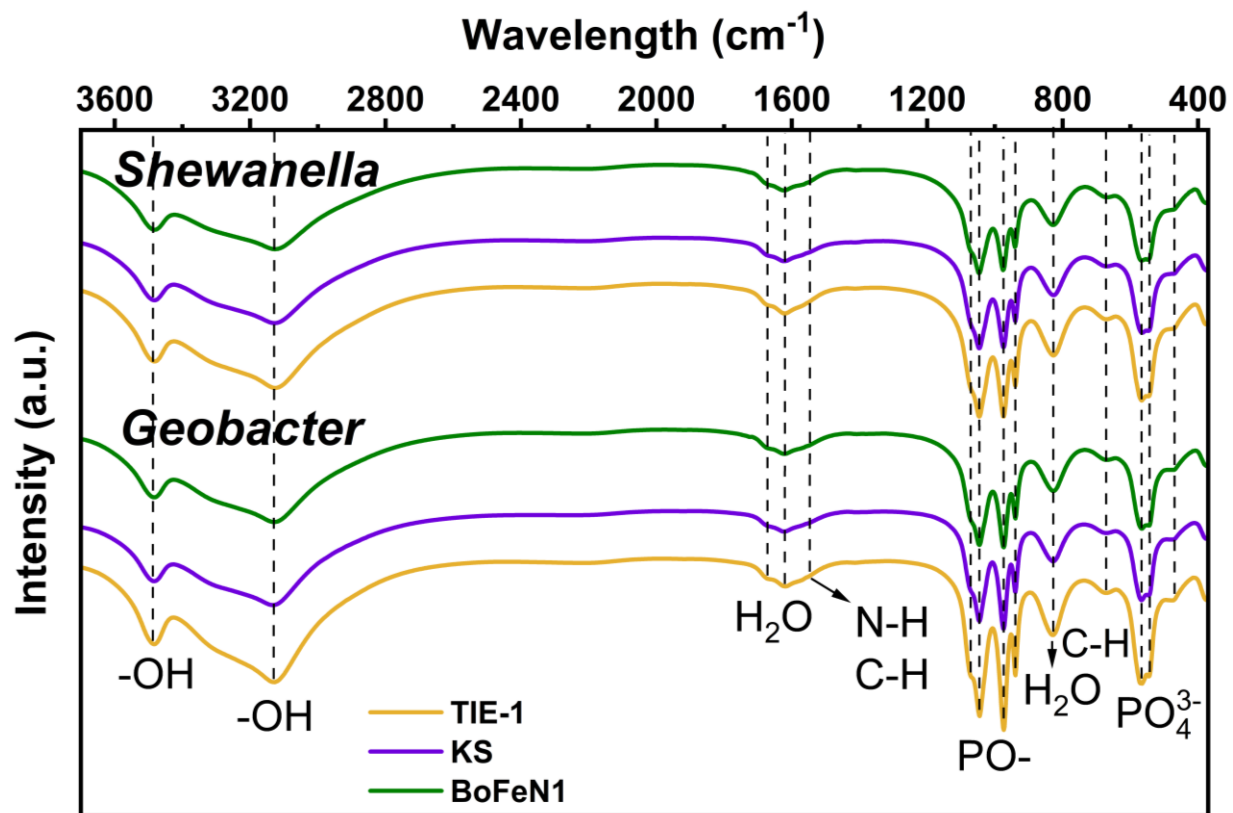


Figure S3. FTIR spectra of the solid phase after reduction of BIOS_{Fe(III)} by *S. oneidensis* (top three) and *G. sulfurreducens* (bottom three). We aimed to elucidate the bonding of the organic carbon within the formed vivianite. However, the spectra showed mainly vivianite peaks, which could be explained by a low organic content. The PO₄³⁻ group is assigned to peaks at 468, 545, 568, 939, 973, 1045 and 1072 cm⁻¹, of which 939, 973 and 1045 cm⁻¹ correspond to the PO stretching.⁷ Water peaks were assigned as follows: peaks at 3460 and 3104 cm⁻¹ correspond to the water OH-antisymmetric stretching modes, that at 831 cm⁻¹ is the vibrational mode and those at 1677 and 1620 cm⁻¹ are the bending bonds.⁸ The organic bonds include the N-H and C-H of amide II,⁹ and the C-H out-of-plane bending vibration at 673 cm⁻¹.¹⁰ The peak at 1548 cm⁻¹ could also be stretching vibrations of carboxyl groups.¹¹

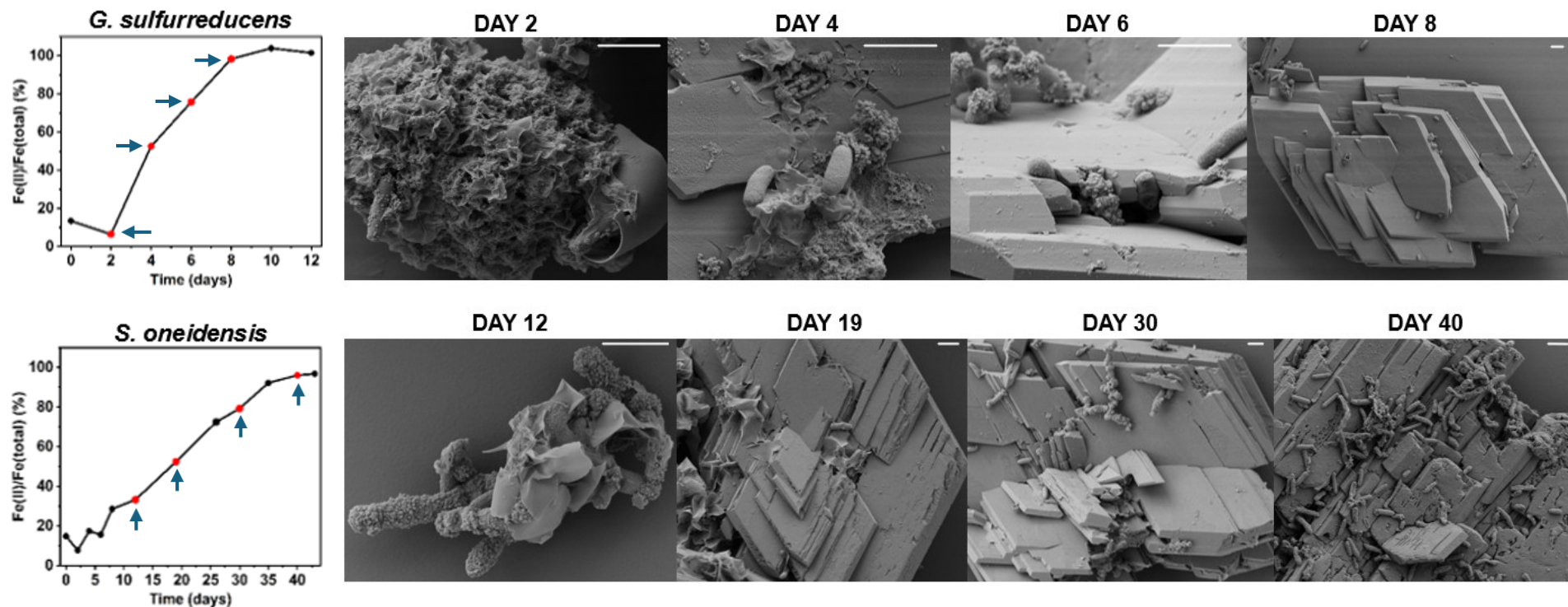


Figure S4. Progressive reduction of BoFeN1 BIOS by *G. sulfurreducens* (top) and *S. oneidensis* MR-1 (bottom). Panels on the left show the Fe(II)/Fe(total) ratio at the sampling point (arrow). After 2 days of incubation with *G. sulfurreducens*, vivianite plates were already forming around Fe(III) (oxyhydr)oxides aggregates. On day 4 when 53% of the initial Fe(III) had been reduced, larger vivianite crystals were observed with cells being partially entombed within them. Some Fe(III) (oxyhydr)oxide aggregates are still observed on this time point. After 76% reduction on day 6, most of the BoFeN1 cells were without Fe(III) coating. Some cells were observed on the surface of the vivianite, while some cells are observed entombed. Similar findings were observed on day 8 when 98% of the initial Fe(III) had been reduced, with vivianite aggregates bigger than 20 μm , some with cells on the surface. With *S. oneidensis*, BoFeN1 BIOS reduction was slower. After 12 days, only 33% of the BIOS_{Fe(III)} had been reduced. However, it can be seen how the transformation occurs around encrusted cells, with smooth thin flakes growing around encrusted cells, yet smaller than the cells at this point. On day 19, when 53% of the initial Fe(III) had been reduced, large vivianite crystals were observed, some containing partially entombed cells. After 30 days when 79% of the Fe(III) had been reduced, the vivianite aggregates were considerably bigger and encrusted cells were still observed on top of the vivianite surface. Almost complete reduction (97%) was achieved on day 40, at which time the majority of the cells were observed on the surface of the vivianite crystal, but without Fe(III) coating. Scale bar indicates 2 μm .

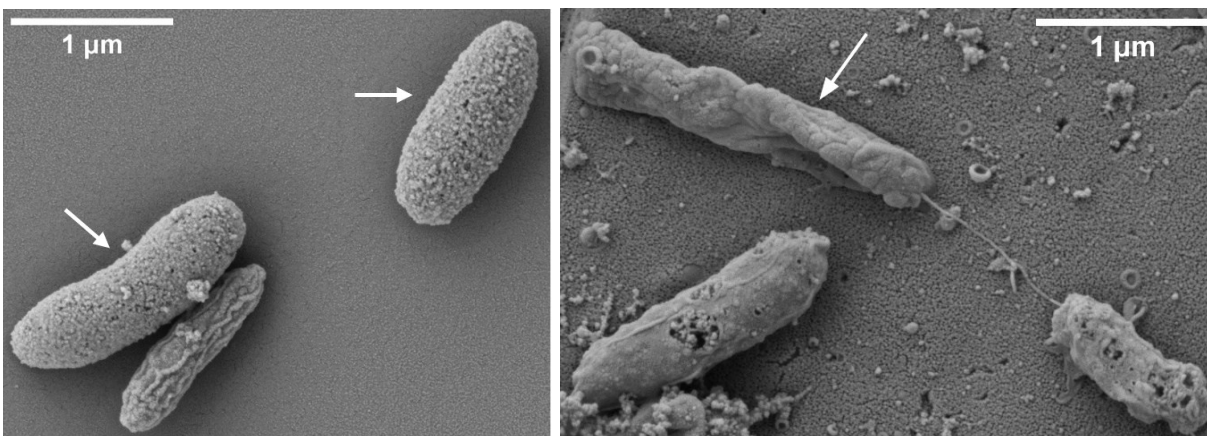


Figure S5. Morphological comparison between (left) BoFeN1 and *G. sulfurreducens*, and (right) BoFeN1 and *S. oneidensis*. (Left) BoFeN1 cells appear thicker and are rod-shaped, typically ~ 1.5 μm long (white arrow). Their surface structures are porous after they lose their Fe(III) (oxyhydr)oxides encrustation. In comparison, *G. sulfurreducens* cells are also rod-shaped but appear thinner and shorter (~ 1.3 μm). Their surfaces commonly exhibit a wrinkled texture. (Right) *S. oneidensis* cells (white arrow) are rod-shaped and longer than BoFeN1 (~ 2 μm). They appear flattened due to poor preservation during SEM preparation. In some cases, pili could be observed. The two cells in the bottom are BoFeN1 cells that appear to be damaged and lysed after losing their Fe(III) (oxyhydr)oxides encrustation.

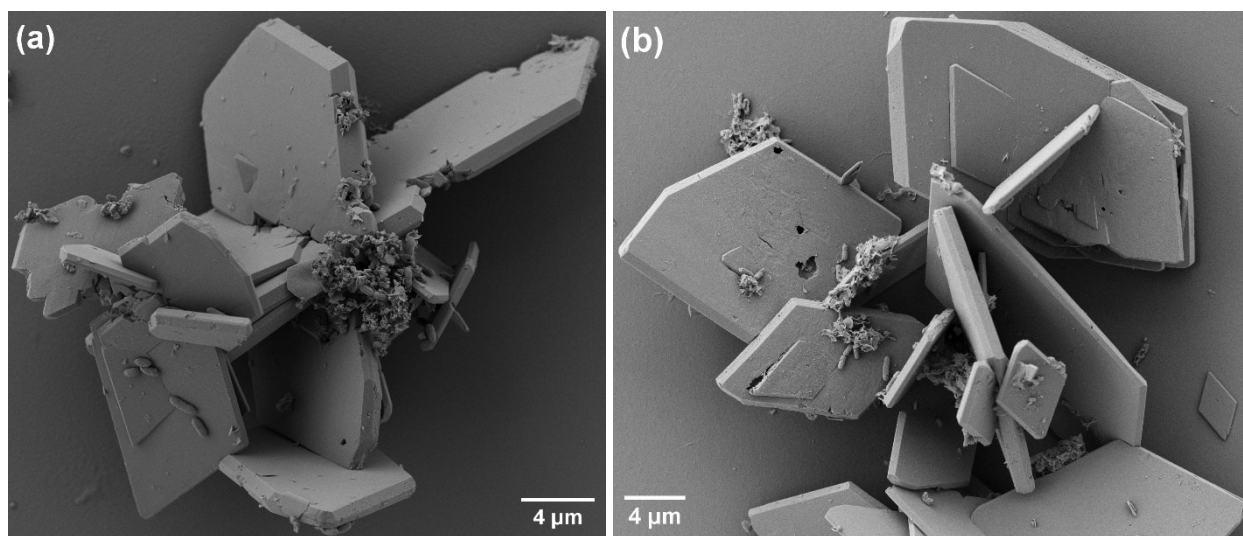


Figure S6. SEM micrographs after reduction of KS BIOS by (a) *G. sulfurreducens* and (b) *S. oneidensis* MR-1. The minerals are mainly flat-plate with sharp edges. The cells are mainly on top of the plates and partial entombment is absent.

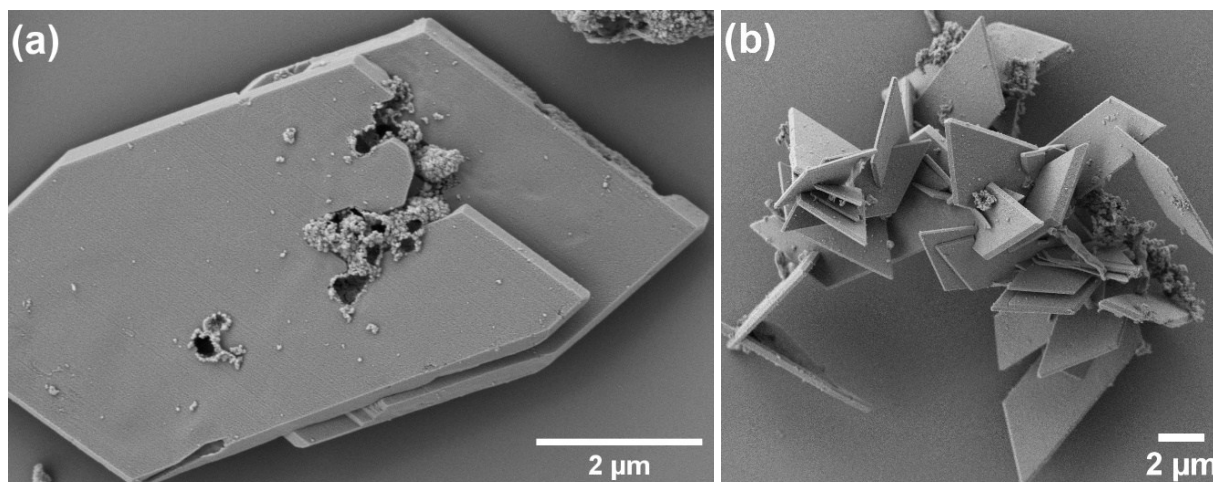


Figure S7. SEM micrographs after reduction of TIE-1 BIOS by (a) *G. sulfurreducens* and (b) *S. oneidensis* MR-1 (bottom). Plates are thinner and sharper than those formed after reduction of KS BIOS. Those formed by *G. sulfurreducens* have holes that resemble those from encrusted cells, although no cell is seen inside. Those formed by *S. oneidensis* aggregated chaotically in random orientations.

Text S1. Particle size and specific surface area (SSA)

Using SEM, we measured the lengths of the vivianite produced after BIOS reduction to be 4-18 μm . The longest vivianite were typically observed to originate from reduction of KS BIOS, followed by BoFeN1, and finally from TIE-1 BIOS. In comparison, Mastersizer-derived sizes of mineral-cell aggregates in suspension ranged from 0.36 to 91.2 μm (mean $\approx 20 \mu\text{m}$), indicating the presence of nanominerals as well as large aggregates. The measured SSA ranged from 2-4 m^2/g , consistent with statistically smaller SSA ($\alpha=0.5$) in incubations with *G. sulfurreducens* compared to *S. oneidensis*.

Table S1. Mean particle size (μm) measured using the Mastersizer, mean particle size (μm) derived from SEM ($n = 13$) and SSA (m^2/g) of the products of reduction

	Mean particle size (μm)		Mean particle length (μm)		Mean particle width (μm)		SSA (m^2/g)	
	Mastersizer		SEM		SEM			
	<i>G. sulfurreducens</i>	<i>S. oneidensis</i>	<i>G. sulfurreducens</i>	<i>S. oneidensis</i>	<i>G. sulfurreducens</i>	<i>S. oneidensis</i>	<i>G. sulfurreducens</i>	<i>S. oneidensis</i>
TIE-1	28.3	20.5	6.46 ± 1.62	5.07 ± 1.52	3.62 ± 1.08	3.01 ± 0.76	2.75	4.07
BoFeN1	24.6	-	14.69 ± 2.51	8.98 ± 2.94	9.49 ± 2.49	4.11 ± 1.52	1.78	4.14
KS	17.4	17.4	15.19 ± 2.79	14.35 ± 2.10	9.69 ± 1.85	10.93 ± 1.29	2.76	4.05

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Chapter 5. Authors contribution

Enrichment of biomass-fermenting microbial consortium and the stability of organic carbon association with biogenic iron (oxyhydr)oxides

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This study was conceptualized by M. Mansor, A. Kappler, J. Lorenz and me. M. Mansor and A. Kappler acquired the funding. The methodology was initially designed by M. Mansor and A. Kappler and later adjusted according to the development of the project by J. Lorenz and me. The enrichments were obtained by J. Lorenz under my supervision and I obtained and carried out the incubations with BIOS. The results of the enrichments were analyzed by J. Lorenz and me, and data visualization was done by J. Lorenz. Data analysis, visualization of the results of incubations with BIOS and writing of the original draft of the manuscript were performed by me. Supervision was provided by M. Mansor and A. Kappler. M. Mansor and A. Kappler contributed to the review and editing of the final manuscript.

Chapter 5. Enrichment of biomass-fermenting microbial consortium and the stability of organic carbon association with biogenic iron (oxyhydr)oxides

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Abstract

Microorganisms capable of organic carbon fermentation are environmentally important as they provide a source of bioavailable organic carbon to drive microbial respiration and carbon cycling. Their metabolisms are also vital for industrial applications to transform carbon waste products into biofuels and valued products. Here, we enriched microbial consortia capable of fermentation from the sediments of Lake Constance, using carbon sources of different complexity: yeast extract, autoclaved biomass of *Shewanella oneidensis* MR-1 and formaldehyde-fixed biomass of *S. oneidensis* MR-1. The respective supplied organic carbon materials were degraded as indicated by continuous decrease in dissolved organic carbon (DOC) and methane accumulation. The main phyla enriched were Bacillota, Bacteroidota, Armatimonadota and Halobacteria, suggesting microbial synergy to degrade different types of organic carbon (e.g., carbohydrates vs proteins). Incubations in the presence of sodium azide (as a respiratory inhibitor) showed decreasing activity with increasing transfer and no methane accumulation, with increasing dominance of Pseudomonadota (from the *S. oneidensis* cells). Therefore, incubations with sodium azide failed to selectively enrich fermentative microorganisms. Since biogenic iron(III) (oxyhydr)oxides (BIOS) are known to sequester organic carbon, we tested their bioavailability to the obtained enrichments. In all cases, neither Fe(III) reduction nor organic carbon degradation was observed. Hence we successfully obtained enrichments that could degrade biomass but not organic carbon associated to BIOS, indicating stability of the latter.

Introduction

Fermentation refers to the transformation process where complex organic compounds are biodegraded to lighter and more bioavailable monomers (e.g. glucose), volatile fatty acids (VFA), methane (CH_4), carbon dioxide (CO_2) and alcohols (e.g. ethanol), among others (De Groof et al., 2019; Stamatopoulou et al., 2020). It occurs naturally in anaerobic environments and is nowadays vital in industrial processes. Fermentation is used as a strategy to overcome climate change and align with the international agreements to decrease the carbon footprint to zero carbon (Gameiro et al., 2016; Halliwell, 1975). It is applied in the biotransformation of organic wastes, from solid waste of the cities and waste waters to food and chemical industry waste (Gameiro et al., 2016; Lim et al., 2008; Mengmeng et al., 2009). Those end-products (commonly considered waste) contain all the necessary compounds (C, N, S, minerals, vitamins) for microbes to thrive and transform them into biofuels and valued chemical products and food (Esercizio et al., 2021; He, 2010; McGovern et al., 2004), replacing the current sources based on fossil hydrocarbons.

The fermentation process is typically divided into two steps: hydrolysis and acidogenesis, and the bacteria involved are jointly termed as fermentative microorganisms (Amin et al., 2021). During hydrolysis, the complex macromolecules (lipids, proteins and carbohydrates) are broken down into single chained fatty acids, amino acids and sugars by hydrolytic bacteria (Amin et al., 2021; He, 2010; Stamatopoulou et al., 2020). Then during acidogenesis, these intermediate compounds are converted into volatile fatty acids (VFAs) by acidogenic bacteria (Amin et al., 2021). Afterwards, two more steps are known during anaerobic digestion (AD): acetogenesis and methanogenesis. AD is the most common application of fermentation in industry, used for the production of biogas (Nguyen and Khanal, 2018; Stamatopoulou et al., 2020). During acetogenesis, acetogenic bacteria transform the intermediate compounds into VFAs, especially acetate. During methanogenesis, the VFAs are transformed into CH_4 , CO_2 , H_2 and water by methanogens. Due to the complexity of the molecules which can be challenging to break down, hydrolysis is the key step (Lowry et al., 1951; Sayara and Sánchez, 2019).

Multiple fermentative consortia, i.e., a community of microorganisms with complementary metabolisms that together carry out fermentation, have been enriched for different objectives. Most of them are enriched for the food industry, for instance to produce kombucha (fermented beverage) or kefir (fermented milk beverage) (Jayabalan et al., 2014; Walsh et al., 2023), as well as for bioprocesses, for instance biofuel production, biohydrogen production or waste water treatment (Hawkes et al., 2007; Janssen et al., 2009; Maeda et al., 2008). Depending on the purpose, the

substrates vary from milk, tea or other biomass (e.g., corn, fruits, among others) in the case of fermented beverages and food (Castellone et al., 2021; Tamang et al., 2016; Walsh et al., 2023), to sludge, manure or biomass (e.g., corn stover, wheat straw, bagasse, wood chips, among others) in the case of bioprocesses (Hawkes et al., 2007; Köpke et al., 2011; Liew et al., 2016). Cellular biomass from plants, animals, or microorganism are used for enriching fermentative microorganisms, due to its richness in macromolecules and the low or moderate need for pretreatment (Markou et al., 2013; Mussnug et al., 2010; Dong et al., 2018). A thermal pretreatment (70–100°C) is sometimes used to enhance its biodegradability (Chen, 1998; González-Fernández et al., 2012). Then, depending on the purpose of the enrichment, inhibitors are used to limit undesired metabolisms. For example, in the production of biohydrogen, VFAs or chain-elongated acids, it is common to use high concentrations of ammonia, 2-bromoethanesulfonate or low pH to inhibit the growth of methanogens (Chen et al., 2008; Rajagopal et al., 2013; Salerno et al., 2006; Sun and He, 2026). Azide, a common inhibitor of the respiratory electron transport and ATP generation is mainly used in laboratory experiments, instead of in large reactors (Keilin, 1936).

In the environment, fermentation is an important process to supply sources of bioavailable carbon. It plays a key role in the carbon cycle, decomposing organic matter in soils and sediments into bioavailable organic substrates (e.g., VFAs) for other organisms (Glodowska et al., 2021; Glombitza et al., 2019; Laloo et al., 2025). Furthermore, this process contributes to the release of greenhouse gases (*i.e.*, CH₄, CO₂). However, the association of carbon to iron (Fe) minerals could inhibit fermentation (or organic matter degradation in general) (Adhikari and Yang, 2015; Lalonde et al., 2012). More than 40% of the organic carbon in soils and 19% in marine sediments is associated to Fe minerals (Jia et al., 2024; Lalonde et al., 2012). Fe minerals can be abiotically formed or form as a result of the oxidation of Fe(II) by Fe(II)-oxidizing bacteria (FeOB), in which case they are known as biogenic iron (oxyhydr)oxides (BIOS). BIOS are mineral-organic aggregates, with the mineral portion consisting typically of ferrihydrite, goethite and amorphous Fe(III) phosphates (Ferris, 2005; Langley et al., 2009; Miot et al., 2009). These minerals are tightly associated with organic matter (OM) and biopolymers, mainly lipid and polysaccharide moieties (Chan et al., 2009; Sowers et al., 2019; Whitaker et al., 2021).

Although the bioavailability of the Fe(III) in BIOS has been studied in the past, little is known about the bioavailability of the BIOS-associated OM. Fermentative microorganisms capable of degrading complex OM could degrade BIOS-associated OM, contributing to the cycling of C even after

interaction with Fe. Therefore, here we attempted to enrich a biomass-fermenting microbial consortium and tested whether it could degrade BIOS-associated OM.

Methods

Sediment collection

Fermentative microorganisms were enriched from sediment taken on February 21, 2024, from the littoral zone of Lake Constance, Germany. Specifically, the sampling sites were on the border of the protected area “Obere Güll” on the Mainau Island (point 1: N47°42'12", E9°11'17", and point 2: N47°42'14", E9°11'19") (**Fig. 1**).

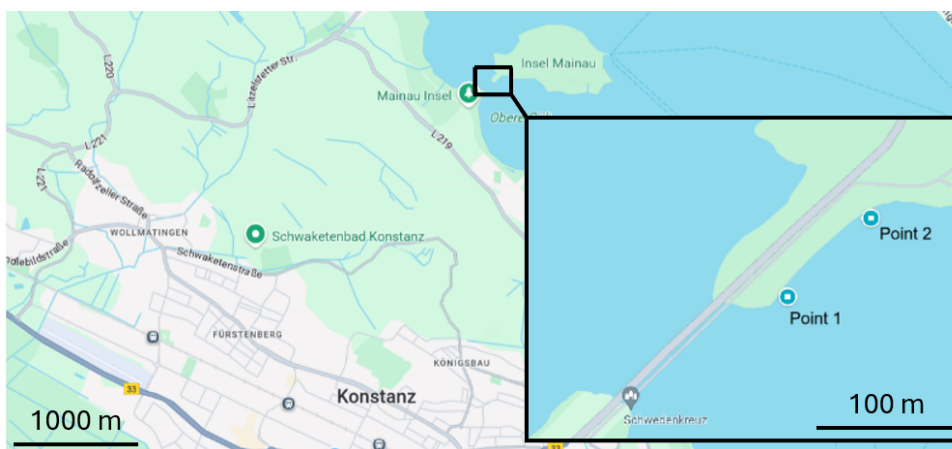


Figure 1. Locations in the littoral zone of Lake Constance, Germany, from where sediment samples were collected (Google Maps).

Sediment samples were stored in sterile Ziploc bags. The sediments were collected at a water depth of ~50 cm and the temperature of the sediment was 8°C and 7.8°C for points 1 and 2, respectively. The temperature and pH of the water at points 1 and 2 were 8.7°C and 8.12, and 8.1°C and 8.24, respectively. Sediment from both locations were mixed in equal proportion prior to their use for the enrichments.

Preparation of carbon sources

Three carbon sources were tested for enrichment of fermentative microorganisms: yeast extract (Yeast), autoclaved biomass of *Shewanella oneidensis* (Auto), and formaldehyde-fixed biomass of *S. oneidensis* (FA fixed) (**Fig. 2**). Biomass was harvested from cells of *Shewanella oneidensis* MR-1. Half of the *S. oneidensis* biomass were grown under anoxic conditions, at pH 7 and 30 °C in Fe(III)-reducer medium amended with 20 mM of sodium-L-lactate and 30 mM of sodium fumarate for three days. The medium was a 30 mM bicarbonate-buffered mineral medium with

0.6 g L⁻¹ KH₂PO₄, 0.3 g L⁻¹ NH₄Cl, 0.5 g L⁻¹ MgSO₄ · 7 H₂O and 0.1 g L⁻¹ CaCl₂ · 2 H₂O, dissolved in ultrapure MQ water and cooled down under a N₂/CO₂ atmosphere after autoclaving (Ehrenreich & Widdel, 1994). Then, sterile trace element solution SL10, vitamin solution (Widdel and Pfennig 1981) and selenite-tungstate solution (Widdel 1980) were added in a concentration of 1 mL L⁻¹ each. The other half was grown oxically at pH 7 and 30 °C in lysogenic broth (LB). LB medium consisted of 10 g L⁻¹ meat peptone, 5 g L⁻¹ yeast extract and 6 g L⁻¹ NaCl dissolved in MQ-water (modified from Myers et al. 1994).

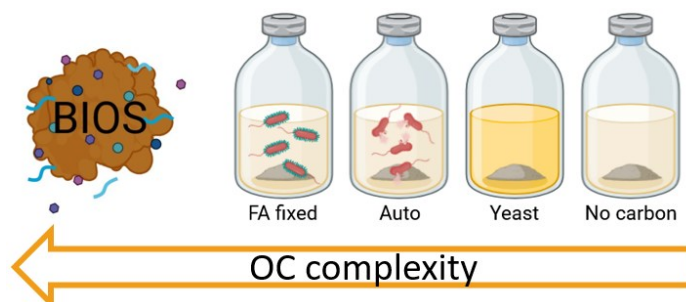


Figure 2. Overview of the setups used for enriching fermenters in this study. It consisted of one setup with only sediment (No carbon) and three setups with sediment amended with 20 mM C from different sources: formaldehyde-fixed biomass (FA fixed), autoclaved biomass (Auto) and yeast extract (Yeast). Four additional setups consisted of the same described above amended with 160 mM azide (NaN₃). Then, successful enrichments from Yeast and FA Fixed were incubated in the presence of BIOS. All setups were done in duplicates.

For the stock of formaldehyde fixed biomass (FA fixed), the cells were treated with 4 % v/v of formaldehyde and stored at 4°C. Optical density was followed over several days to ensure no further growth happened. Then, it was washed with freshwater medium by centrifugation at 5000 g for 5 min, discarding the supernatant and redissolving the pellet four times. The final DOC concentration in the stock was 532 mM C as measured by TOC analyzer.

For the stock of autoclaved biomass, the stock was autoclaved two times. Due to the formation of particulates after autoclaving, the stock was sonicated in multiple steps for a total time of 1 h 10 min, although complete dissolution of the particles was not achieved. The final DOC concentration in the stock was 638 mM C as measured by TOC analyzer.

The stock solution of yeast was prepared by diluting 2.4 g of yeast extract in 25 ml of MQ-water. The solution was flushed with N₂ in a serum bottle and closed anoxically with a butyl stopper.

Then the solution was autoclaved. The dissolved carbon concentration was 3794 mM C as measured by TOC analyzer.

Enrichment of fermentative microorganisms

All glassware was washed with HCl and baked at 300°C for 8 h before use. The experiment consisted of 8 different setups all in duplicates, six with 20 mM of dissolved organic carbon and two without additional carbon sources. Half of the setups (one of each carbon source and one without) were amended with sodium azide (NaN_3) to a final concentration of 160 mM, as a respiratory inhibitor to specifically target enrichment of fermentative microorganisms. For the first inoculation (transfer 1), approximately 2 g of sediment were weighted into 100 ml serum bottles under anoxic conditions and closed air-tight with butyl stoppers. Then, 50 mL of freshwater medium were added, along with the respective carbon source (Yeast, Auto or FA-Fixed). Freshwater medium was a 30 mM bicarbonate-buffered medium prepared by diluting 1 g L⁻¹ NaCl, 0.4 g L⁻¹ $\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$, 0.1 g L⁻¹ $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$, 0.25 g L⁻¹ NH_4Cl , 0.2 g L⁻¹ KH_2PO_4 and 0.5 g L⁻¹ KCl and 2.5 g L⁻¹ of NaHCO_3 in MQ-water (modified from Widdel and Bak 1992). Then, autoclaving, cooling under N_2/CO_2 atmosphere and adding sterile trace element solution SL10, vitamin solution (Widdel and Pfennig 1981) and selenite-tungstate solution (Widdel 1980) in a concentration of 1 mL L⁻¹ each. The pH was adjusted to 7 ± 0.1 with sterile and anoxic 1 M HCl. NaN_3 was added afterwards to the respective treatments. The headspace of all bottles was flushed at the end with N_2/CO_2 to ensure anoxic starting conditions. The following transfers were inoculated with 5% v/v inoculum from the respective previous transfer.

Sampling and geochemical analysis

Sampling was performed at least every 7 days, depending on the setup. Samples were collected in a glovebox (100% N_2) with a 1 ml syringe and placed into two Eppendorf tubes, 1 ml each. From one tube, 150 µl were transferred to a 600 µl Eppendorf tube containing 150 µl of 1 mM HCl for Fe total and Fe(II) concentration measurements (by ferrozine assay). The other container was centrifuged at 12045 g for 5 min. From the supernatant, 300 µl were transferred to a glass vial containing 1.2 ml of 80 mM HCl for dissolved organic carbon (DOC) analysis and 150 µl were added to a 600 µl Eppendorf tube containing 150 µl of 1 mM HCl for Fe^{2+} concentration measurements.

Dissolved Organic Carbon (DOC) was measured in triplicates with a TOC analyzer (multi N/C 2100S, Analytik Jena AG, Germany). Each run contained standards of 5, 10, 20 and 40 mg/L

organic carbon for calibration. Fe content was quantified in triplicates by colorimetry using the ferrozine assay (Hegler et al., 2008; Stookey, 1970). In all cases, 20 µl of sample were added to 80 µl of either 1 M HCl (for Fe(II)) or 10% w/v hydroxylamine hydrochloride in 1 M HCl (for Fe total), in a 96-well plate. After 30 min of reaction, 100 µl of ferrozine reagent were added to all wells and left incubating during 5 min. Then, the ferrozine-Fe(II) complex was measured at 562 nm in a plate reader (Multiskan GO; Thermo Fisher Scientific, USA). Each 96-well plate also contained triplicates of 50, 100, 200, 400, 600, 800, and 1000 µM Fe(II) ethylenediammonium sulfate tetrahydrate for standard curve calibration.

Gas samples were measured by gas chromatography (Thermo Fisher Scientific Trace GC Ultra) with a thermal-conductivity detector. The column was a silica capillary 30 m × 0.25 mm × 0.25 µm and helium was the carrier gas at 10 mL/min. Of each sample, 100 µl were injected.

16S rRNA Illumina Sequencing

DNA for 16S rRNA Illumina Sequencing was extracted from the original sediment and at the end point of transfers 1 to 3 using the DNeasy PowerSoil Pro Kit (Qiagen). After extraction, a polymerase chain reaction (PCR) was carried out to increase DNA content. For this, the forward primer was 515f PCR primer sequence, and the reverse primer sequence was 806r PCR primer (Caporaso et al. 2011). DNA extracts were amplified in 35 cycles using a Mastermix (**Table 1**). DNA content for sequencing was checked by gel electrophoresis on agarose gel. Samples were sent to the NGS Competence Center Tübingen (NCCT), Germany for 16S rRNA Illumina Sequencing. Downstream analysis was done by the Quantitative Biology Center (QBiC), Tübingen, Germany.

Table 1. Mastermix recipe for Illumina PCR.

Component	Volume (µl)
Microbial DNA	5
Amplicon PCR Forward 10 µM	0.3
Amplicon PCR Reverse 10 µM	0.3
2 x KAPA Hifi HotStart Ready Mix	12.5
RNAse/DNAse free water	6.9

Bioinformatic analysis

The nfcore/ampliseq pipeline was used for the analysis of the sequencing (Ewels et al. 2020; Straub, Blackwell, et al. 2020). First, the quality of the sequence was checked using FastQC.

Then, the resulting data was aggregated with the FastQC module of MultiQC. Primer sequences were trimmed using cutadapt and the untrimmed sequences were discarded. DADA2 was used for further quality filtering, PCR chimera removal, and denoising. Genome Taxonomy Database (GTDB) and SILVA were the reference databases for the sediment samples and the enrichment culture samples, respectively.

BIOS sources

BIOS were obtained from enrichment culture KS, where BIOS are the result of chemoautotrophic Fe(II) oxidation coupled to nitrate reduction. Culture KS was grown anoxically in 100 ml serum bottles with 50% v/v 22 mM bicarbonate-buffered mineral medium at pH 7 (Ehrenreich & Widdel, 1994). The headspace was a 90:10% v/v mix of N₂/CO₂ and the bottles were placed in the dark at 28°C. The media was amended with 10 mM Fe(II) (as Fe₂Cl) and 2 mM NO₃⁻ (as NaNO₃). The inoculum was 1% (vol/vol) of a preculture and the precultures were grown with the same conditions as described above. BIOS were collected by centrifugation (12045 g for 2 minutes and the supernatant was exchanged with fresh FeRB medium). FeRB medium consisted of KH₂PO₄ (4.41 mM), NH₄Cl (5.61 mM), MgSO₄ 7H₂O (2.00 mM), CaCl₂ 2H₂O (0.68 mM), NaHCO₃ (30 mM), Trace elements SL10 (1 ml/L), 7 Vitamine solution (1 ml/L) and Selenite/Tungstate solution (1 ml/L). BIOS collection was performed when 93±0.2% Fe(II) was oxidized, as tracked by the ferrozine assay.

Incubation of fermentative microorganisms with BIOS

The degradation of the OM associated to BIOS was tested with fermentative microorganisms from: a) the transfer 5 of setups Yeast and FA fixed with NaN₃ and b) the transfer 6 of setups Yeast and FA fixed without NaN₃. For this, the setups were amended with 6 mM total Fe from BIOS as sole OM source and an inoculum of 5% v/v of the corresponding enrichment was added. This amount of BIOS_{Fe(III)} corresponded to 0.15 mM of BIOS-associated OM, considering the total organic carbon content was 0.24 wt% as determined with a soliTOC (Cube, Elementar, Germany).

Results

Microbial community of the original sediment

The two replicates of the original sediment contained 76 phyla, among which 9 and 10 phyla had abundances >1% in replicates a and b, respectively (**Fig. 3**). The phyla Pseudomonadota (36.33 ± 1.10 %) and Cyanobacteriota (21.05 ± 1.21 %) were the most abundant, followed by Chloroflexota (12.58 ± 0.02%), Bacteroidota (9.87 ± 0.69%), Acidobacteria (6.88 ± 0.37%),

Verrucomicrobiota ($5.63 \pm 0.17\%$), Actinomycetota ($5.08 \pm 0.39\%$), Planctomycetota ($6.18 \pm 0.28\%$) and Desulfobacterota ($3.47 \pm 0.07\%$).

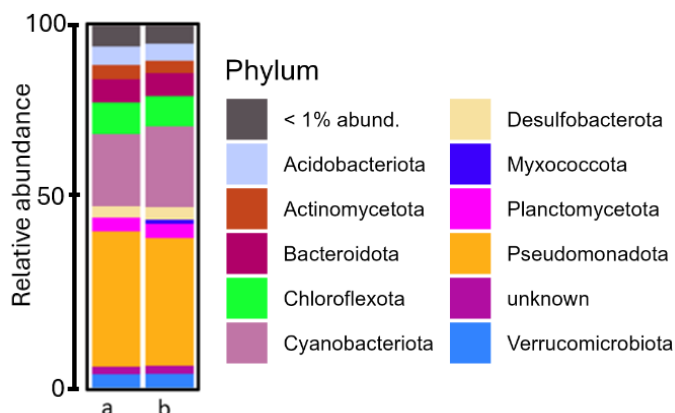


Figure 3. Relative abundance of phyla in the original sediment.

Enrichment with biomass of different complexity

Microbial diversity

Using biomass with different complexities, there was a significant change in the phyla abundances compared to the sediment. Initially low-abundant phyla were enriched, similar phyla among the different setups.

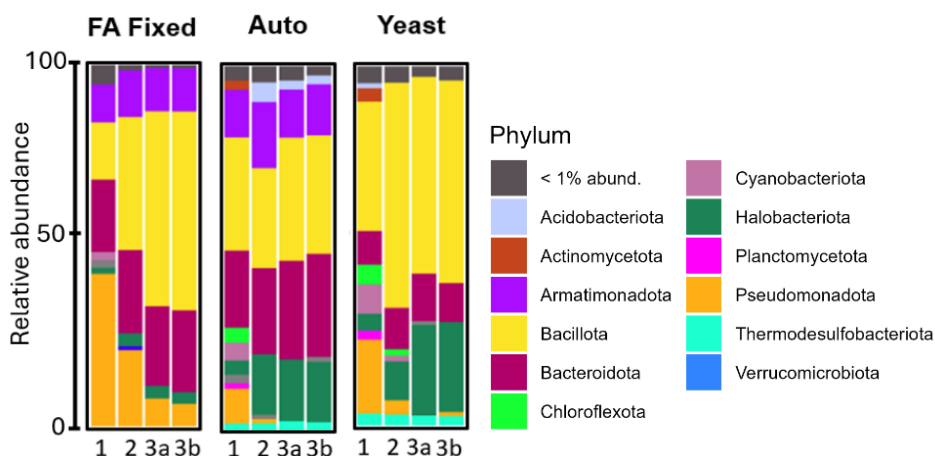


Figure 4. Relative abundance of phyla enriched over the first three transfers using formaldehyde-fixed biomass (FA-Fixed), autoclaved biomass (Auto) and Yeast as carbon source.

With FA-Fixed biomass (the most complex carbon source used), four main phyla were enriched through all the transfers: Pseudomonadota, Bacillota, Bacteroidota and Armatimonadota (**Fig. 4**). From transfer 1 to 3, the richness decreased from 44 phyla to 18 and 17 in replicates 1 and 2, respectively. In the last transfer, the main phyla were Bacillota (54% and 55%), Bacteroidota (22%

and 23%), Armatimonadota (12%), and Pseudomonadota (8% and 6%). *S. oneidensis*, used as biomass, decreased in abundance from 37.2% at the end of transfer 1, to 7.4% and 6.1% at the end of transfer 3.

In the enrichment with autoclaved biomass (Auto), already at the end of the first transfer there were three phyla that dominated the enrichment, the same enriched with FA-Fixed, and Pseudomonadota was less abundant. Then, from transfer 2 on, Halobacteria was also enriched and Pseudomonadota was lost. Through the transfers, the richness also decreased, from 53 in transfer 1, to 26 and 24 phyla in transfer 3 in replicates 1 and 2, respectively. In the last transfer, Bacillota was the most abundant (34% and 33%), followed by Bacteroidota (24%), followed by Halobacteriota, and Armatimonadota (13% and 14%).

Finally, when using yeast as carbon source, the richness decreased from 54 in transfer 1, to 36 and 38 different phyla in replicates 1 and 2 of transfer 3, respectively. At the end of transfer 3, the main phyla were Bacillota (55% and 56%), Halobacteriota (25%) and Bacteroidota (13% and 11%).

Aqueous chemistry

In the setups with FA-Fixed and Auto, the aqueous chemistry was similar. With FA-Fixed, initially there was an increase in the DOC concentration (~9 mM), from initial DOC concentrations of 3.61 ± 0.22 mM in transfer 1, 2.45 ± 0.02 mM in transfer 2, and 2.38 ± 0.03 mM in transfer 3, to a maximum concentration of 15.58 ± 0.26 mM on day 28 in transfer 1, 10.37 ± 0.20 mM on day 76 in transfer 2, and 10.21 ± 0.39 mM on day 113 in transfer 3. After this increase, the DOC concentration decreased close to its initial value (**Fig. 5a**).

With Auto, initially there was an increase in DOC concentration (~5 mM), from 13.32 ± 1.49 mM in transfer 1, 9.94 ± 0.07 mM in transfer 2, and 8.86 ± 0.07 mM in transfer 3, to a maximum concentration of 17.74 ± 0.30 mM on day 21 in transfer 1, 15.68 ± 0.03 mM on day 70 in transfer 2, and 13.78 ± 0.06 mM on day 103 in transfer 3. Afterwards, there was a decrease to a final concentration of 2.90 ± 0.42 mM, 2.24 ± 0.11 mM, and 2.31 ± 0.04 mM, for transfers 1, 2 and 3, respectively (**Fig. 5b**).

Finally, with yeast, there was complete DOC consumption in all transfers, which was expected as yeast is widely used as carbon source due to its high bioavailability (**Fig. 5c**).

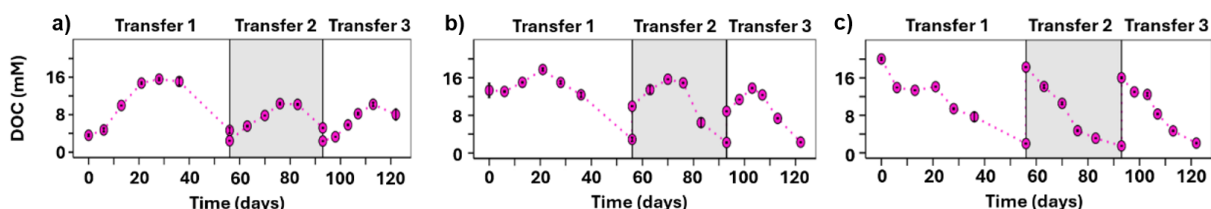


Figure 5. Aqueous chemistry (DOC) over the first three transfers using (a) formaldehyde-fixed biomass, (b) autoclaved biomass and (c) yeast as carbon source.

Gas concentration

Overall, the concentration of CO_2 initially increased and then decreased, while CH_4 increased within each transfer in all setups.

With FA-Fixed, in transfer 1, the CO_2 increased to a maximum of 46096 ± 11762 ppm on day 21 and then decreased to 15026 ± 854 ppm on day 56. The CH_4 concentration increased to the highest concentration of 60383 ± 2432 ppm on day 56. In transfer 2, there was a steady increase in CO_2 to a maximum of 30545 ± 9083 ppm on day 93. The CH_4 concentration increased to its highest concentration of 47700 ± 637 ppm on day 93. In transfer 3, the CO_2 concentration fluctuated until day 113, when it decreased to -3059 ± 977 ppm (below the initial concentration). The CH_4 concentration increased to the highest concentration of 29269 ± 1384 ppm on day 122. In all transfers, the H_2 concentration first increased and then decreased. For transfers 1, 2 and 3 the highest concentration was 352 ± 10 ppm on day 13, 395 ± 4 ppm on day 70 and 984 ± 28 ppm on day 103, respectively (**Fig. 6a**).

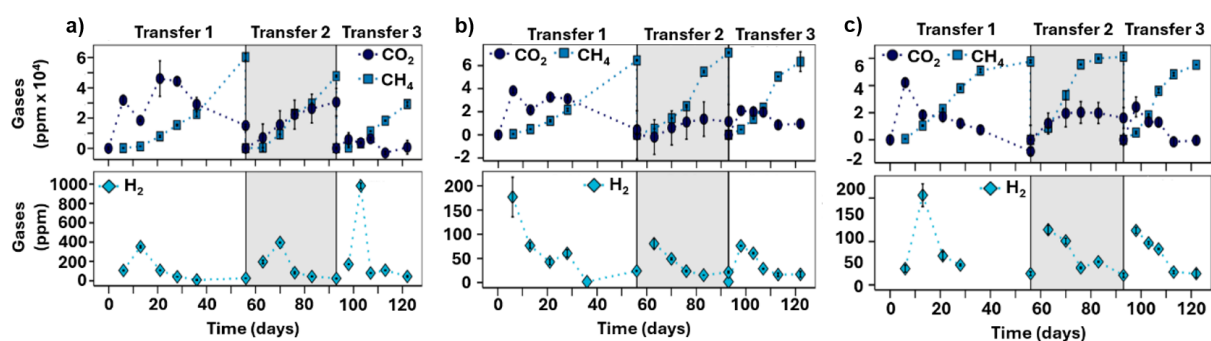


Figure 6. Gas concentration over the first three transfers using (a) formaldehyde-fixed biomass, (b) autoclaved biomass and (c) yeast as carbon source.

With Auto, the ΔCO_2 did not have the same trend in all transfers, similar to that in FA-Fixed. In transfer 1, it increased to 38070 ± 1938 ppm on day 6, to then decrease to 4470 ± 3100 ppm on

day 56. In transfer 2, the CO₂ concentration increased overall. Then in transfer 3, it increased to a concentration of 20996 ± 4048 ppm on day 98 to then decrease after day 113 to a final concentration of 9627 ± 3423 ppm on day 122. In the case of CH₄, it increased in all the transfers, to a maximum concentration of 64526 ± 462 ppm on day 56, 71197 ± 633 ppm on day 93, and $63\,415 \pm 8571$ ppm on day 122, for transfer 1, 2 and 3, respectively. In the case of H₂, it increased quickly to its maximum within the first 6 days of each transfer, to 177 ± 41 ppm on day 6, 80 ± 11 ppm on day 63, and 76 ppm on day 98 for transfers 1, 2 and 3, respectively. It then decreased in all transfers (**Fig. 6b**).

When using yeast as carbon source, in transfers 1 and 3, the CO₂ concentration increased and then decreased, while in transfer 2 it increased to an almost steady state. The maximum concentration in transfers 1, 2 and 3 was 42118 ppm on day 6, 20229 ± 7965 ppm on day 76 and $24\,226 \pm 7282$ ppm on day 96, respectively. Then transfers 1 and 3 decreased to -8473 ± 2602 ppm on day 56 and -377 ± 3508 ppm on day 122, respectively. In all transfers, the CH₄ concentration increased continuously. The maximum concentration was 57713 ± 919 ppm on day 56, 61252 ± 1174 ppm on day 93, and 55279 ± 53 ppm on day 122, at the end of each transfer, respectively (**Fig. 6c**).

Enrichments amended with azide (NaN₃)

Microbial diversity

In all cases, NaN₃ caused a faster loss of diversity along the transfers (**Fig. 7**). Furthermore, in all cases, the most abundant phylum enriched was Pseudomonadota.

With FA-Fixed, the enrichment of Pseudomonadota was more pronounced, increasing from 79.0% to 98.8% (from transfer 1 to 3). The richness decreased from 43 phyla to 21 and 17 in replicates 1 and 2, respectively. Within Pseudomonadota, *S. oneidensis* was the most abundant species, with 98.9% and 98.8% in replicates 1 and 2, respectively, in transfer 3.

In the case of Auto, a total of 53 phyla were detected in transfer 1, and it decreased to 43 and 42 in transfer 3 in replicates 1 and 2, respectively. Pseudomonadota was the most abundant, phylum with 88% and 91%, in replicates 1 and 2, respectively, in transfer 3. Within this, 86.8% and 80.9% was *S. oneidensis* in replicates 1 and 2, respectively. All the other phyla had abundances below 5%.

In the setup with yeast, the richness decreased from 50 to 44 in transfer 2. The sequencing data from both samples of transfer 3 were excluded because they did not yield well enough reads to be analyzed. Pseudomonadota was the most abundant phylum and increased its abundance from 37% to 39%. In transfer 2, Pseudomonadota (0.6%) was followed by Actinomycetota (18%), Chloroflexota (12%), and Cyanobacteriota (10%).

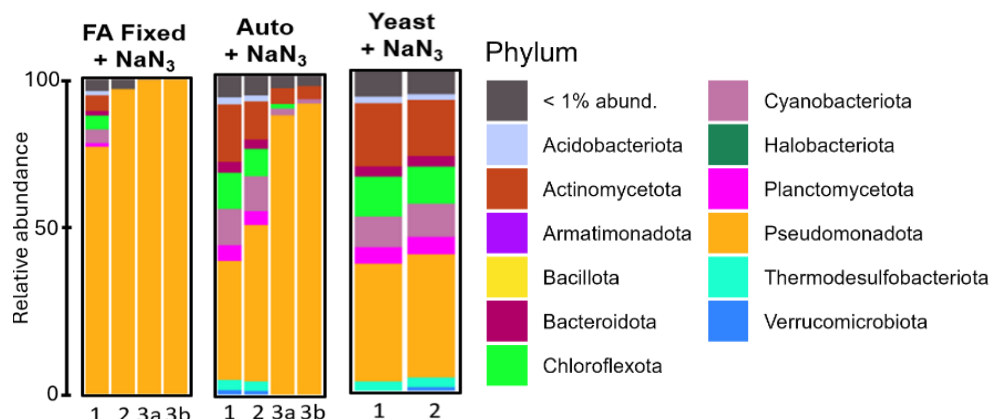


Figure 7. Relative abundance of phyla enriched over the first three transfers using formaldehyde-fixed biomass (FA fixed), autoclaved biomass (Auto) and Yeast as carbon source in the presence of NaN_3 .

Aqueous chemistry

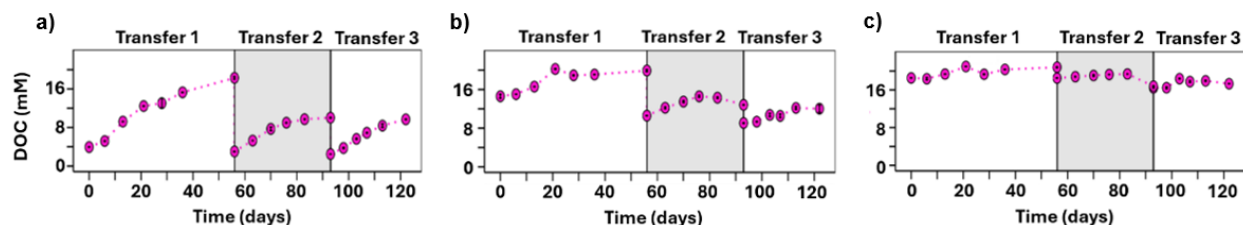


Figure 8. Aqueous chemistry (DOC) over the first three transfers using (a) formaldehyde-fixed biomass, (b) autoclaved biomass and (c) yeast as carbon sources in the presence of NaN_3 .

With FA-Fixed there was a significant increase in the DOC concentration. Given that Pseudomonadota is the most abundant and almost only phylum, the increase in the DOC could be abiotic degradation of the cells by the formaldehyde, favored by the temperature at which the enrichments were grown (Carson and Cappellano, 2015; Ortega-Atienza et al., 2016). From an initial concentration of 3.95 ± 0.01 mM in transfer 1, 3.04 ± 0.04 mM in transfer 2, and 2.50 ± 0.01 mM in transfer 3, it increased continuously to a maximum of 18.29 ± 0.26 mM on day 56, 10.04 ± 0.08 mM on day 93, and 9.69 ± 0.07 mM on day 122, in transfers 1, 2 and 3, respectively (**Fig. 8a**). In the setups with autoclaved biomass, there was a slight increase in DOC, although there was no

decrease afterwards (**Fig. 8b**). In the presence of NaN_3 , there was no significant difference in the DOC with yeast as carbon source (**Fig. 8c**).

Gas concentration

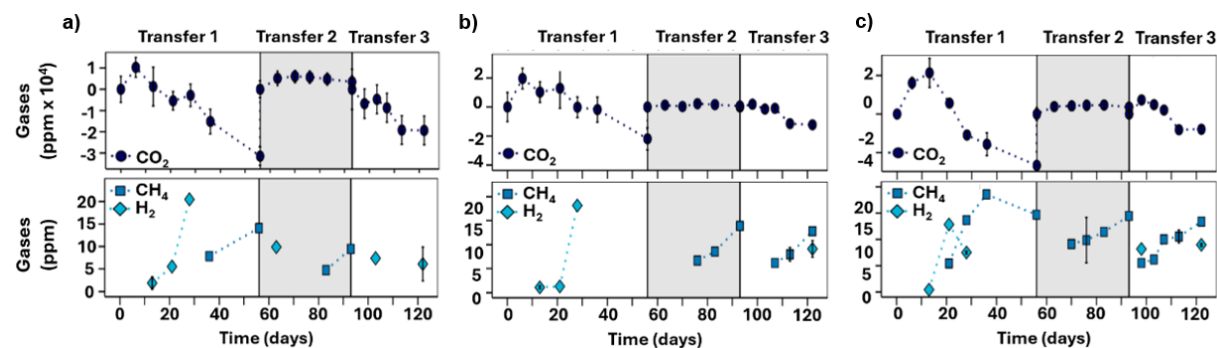


Figure 9. Gas concentration (CO_2 , CH_4 and H_2) over the first three transfers using (a) formaldehyde-fixed biomass, (b) autoclaved biomass and (c) yeast as carbon sources in the presence of NaN_3 .

In the FA-Fixed setup, the CO_2 concentration decreased overall after a slight increase of 10308 ± 4646 ppm on day 6 and 6000 ppm on days 70 and 93. In transfer 2, this value was steady, while in transfers 1 and 3 it decreased to -31453 ± 4323 ppm on day 56 and -19348 ± 6753 ppm on day 122, respectively (**Fig. 9a**). In the case of CH_4 and H_2 , there was no real trend and multiple times the values were below the limit of detection. With Auto, transfer 1 showed a slight increase in CO_2 concentration the first 5 days, and then decreased to a final concentration of -21885 ± 7632 ppm on day 56. In transfers 2 and 3 the concentration remained unchanged, except for a decrease on day 107 (transfer 3) to -12235 ± 1666 ppm (**Fig. 9b**). As with FA-Fixed, the CH_4 and H_2 did not show a trend and several times the values were below the limit of detection. Finally with yeast, the CO_2 concentration had a similar behavior than with Auto, but more pronounced. In transfer 1 there was an increase of 21320 ± 7551 ppm on day 13 and in transfer 3 of 7302 ± 1839 ppm on day 98, and then a decrease to -26638 ± 170 ppm and -7896 ± 1763 ppm on the last day of each transfer, 56 and 122, respectively. In transfer 2, the CO_2 concentration remained almost unchanged (**Fig. 9c**). Although there is increase in CH_4 concentration, it is low (~ 10 ppm) and interpreted as a product of abiotic reactions of the NaN_3 in the carbonate medium, considering that the sequencing data does not show presence of Halobacteria.

Enrichment without additional carbon

When enriched without additional carbon sources, *Pseudomonadota* remained the most abundant phylum in both treatments (with and without azide) (**Fig. 10**).

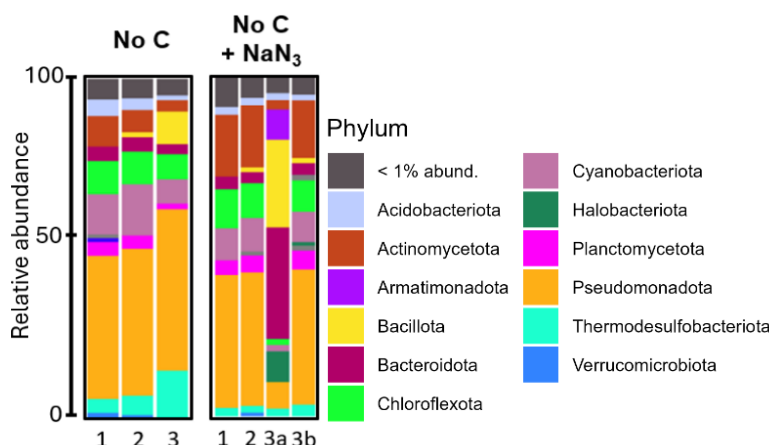


Figure 10. Relative abundance of phyla enriched without additional carbon sources in the absence (left) and presence (right) of NaN_3 .

Without NaN_3 , richness decreased from 53 phyla in transfer 1, to 46 in transfer 3. *Pseudomonadota* was the most abundant phylum with 42%, followed by *Cyanobacteriota* (12%), *Chloroflexota* (10%), and *Actinomycetota* (9%), while other phyla had abundances below 5%, in transfer 1. Then, in transfer 3, *Pseudomonadota* was still the predominant phylum and increased its abundance to 48%, followed by *Thermodesulfobacteriota* (14%), *Bacillota* (10%), *Chloroflexota* (~7%) and *Cyanobacteriota* (~7%).

In the setup with NaN_3 , the first transfer also yielded 53 phyla, which then reduced to 45 and 38 in the two replicates of transfer 3, respectively. The most abundant phyla in transfer 1 were *Pseudomonadota* (39%), *Actinomycetota* (18%), *Chloroflexota* (12%), and *Cyanobacteriota* (9%). Then, in transfer 3, the two replicates yielded different enrichments. Replicate 1 enriched *Bacteroidota* (33%), *Bacillota* (26%), followed by *Halobacteriota* (9%), *Armatimonadota* (9%) and *Pseudomonadota* (8%), while replicate 2 enriched, *Pseudomonadota* (40%), *Actinomycetota* (17%), *Chloroflexota* (9%), *Cyanobacteriota* (9%), and *Planctomycetota* (6%).

Aqueous chemistry and gas concentration

In both treatments, the concentration of DOC remained without considerable changes (**Fig. 11**). The CO_2 concentration tended to decrease in transfers 1 and 3, while transfer 2 remained almost unchanged. In transfers 1 and 3 without NaN_3 , it increased to 15383 ± 3408 ppm on the first 6

days after the first transfer and then decreased to -27227 ± 4467 ppm on day 56 and to -22185 ± 2764 ppm on day 122. In transfers 1 and 3 with NaN_3 , it increased to ~ 13 ppm on day 6 and then decreased to -28959 ± 3155 ppm on day 56 and to $-30\,169 \pm 14\,963$ ppm on day 122. In transfer 2, the concentration remained between 4195 ± 2646 ppm and 2243 ± 2680 ppm without NaN_3 and -354 ± 1435 and -2461 ± 1456 ppm with NaN_3 . The CH_4 showed increase only in the absence of NaN_3 , although the extent decreased over the three transfers, reaching ~ 2000 ppm in the first transfer, but ~ 100 ppm in transfer 2 and ~ 20 ppm in transfer 3. The H_2 remained unchanged and close to 0 in the first transfer in the absence of NaN_3 and decreased from ~ 20 ppm to close to 0 in transfers 2 and 3. In the presence of NaN_3 there was no trend and most of the time it was below the detection limit.

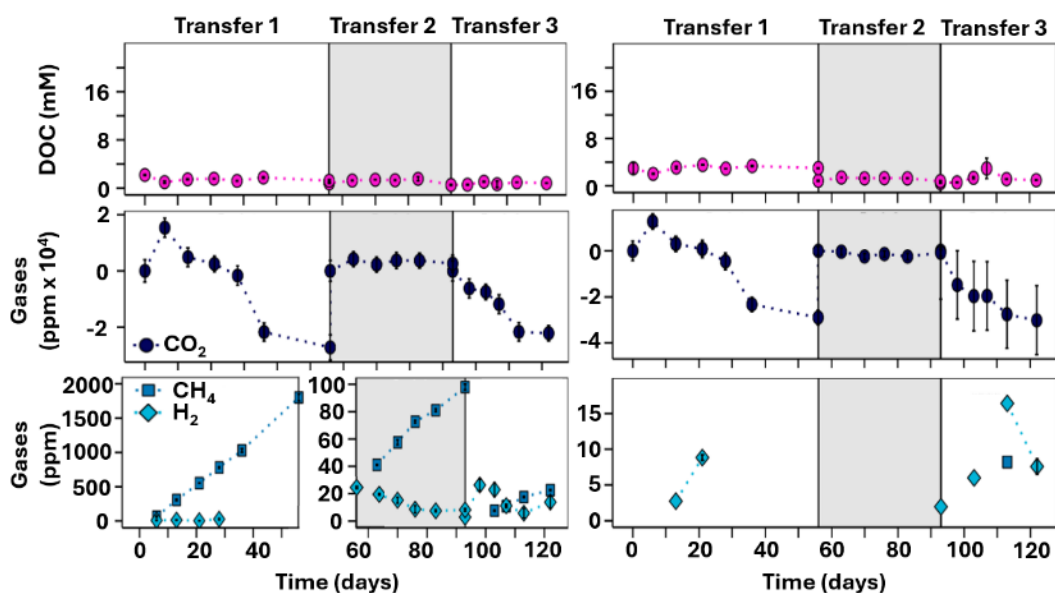


Figure 11. Aqueous chemistry and gas concentration over the first three transfers for enriching fermentative microorganisms from the sediment without additional carbon sources in the absence (left) and presence (right) of NaN_3 .

BIOS-OM degradation

Based on the aqueous chemistry and the changes in the gases concentration we chose the enrichments from the treatments FA-Fixed and Yeast (with and without NaN_3) for fermentation of the BIOS-OM. Using BIOS from culture KS, after 90 days of incubation there was Fe(III) reduction with the enrichment from FA-fixed without NaN_3 (**Fig. 12a**), while there was no significant increase in the Fe(II) concentration in the setup with NaN_3 (**Fig. 12b**).

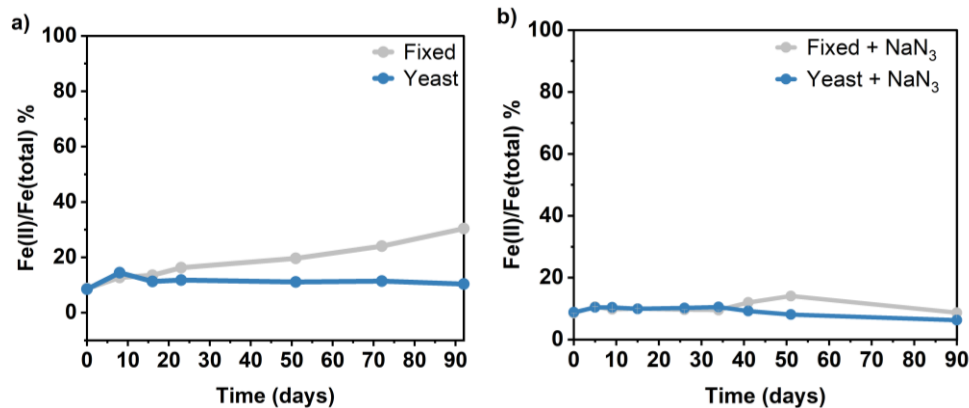


Figure 12. Fe(II)/Fe(total) ratio of the incubation of fermentative microorganisms with BIOS of culture KS with enrichments (a) without and (b) with NaN₃. Symbols are the media of 2 replicas and error bars were hidden by the symbols.

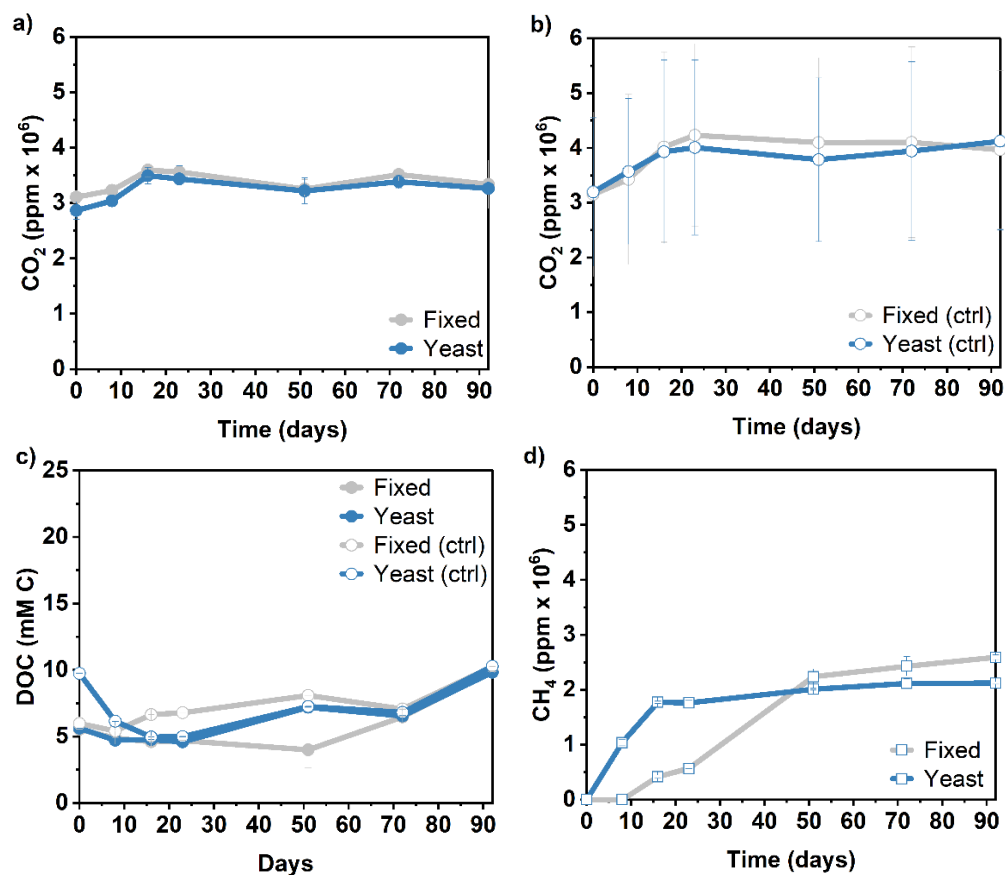


Figure 13. Concentration of CO₂ in enrichments with Yeast and FA-fixed grown with (a) culture KS BIOS as sole carbon source and (b) the corresponding carbon source (control). (c) DOC concentration in the setups with BIOS (full symbols) and the controls (empty symbols). (d) Concentration of CH₄ in the controls.

In the setups with enrichments without NaN_3 , there was increase in the CO_2 concentration ($\sim 0.5 \times 10^6$) and $\sim 0.9 \times 10^6$ ppm in the control (**Fig. 13a-b**). The DOC concentration was similar in the experiments and the controls, with an increase of 5 mM, except in the control of Yeast, where the increase was only ~ 0.5 mM, although the trend in all of them was similar from day 8 on (**Fig. 13c**). Finally, production of CH_4 occurred only in the controls, increasing $2.1 \times 10^6 \pm 2.1$ ppm with Yeast and $2.6 \times 10^6 \pm 2.6$ with FA-Fixed (**Fig. 13d**).

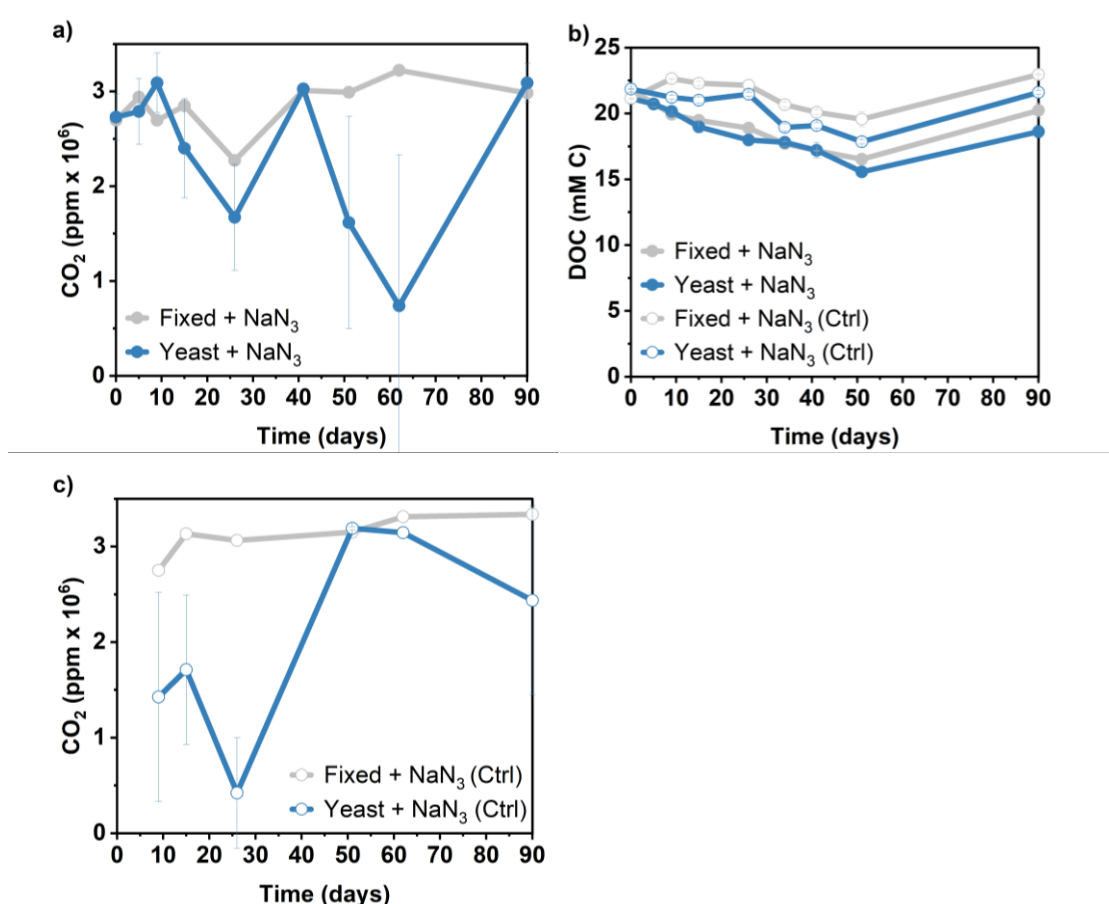


Figure 14. Concentration of CO_2 (a) and DOC (b) in enrichments with Yeast + NaN_3 and FA-Fixed + NaN_3 grown with culture KS BIOS as sole carbon source (full symbols) and the corresponding carbon source (empty symbols). (c) Concentration of CO_2 in the controls.

The CO_2 concentration in the setup with Yeast+ NaN_3 enrichment oscillated, with minima after 25 and 61 days, the second more prominent than the first one ($1.7 \times 10^6 \pm 0.5$ and $\sim 0.7 \times 10^6 \pm 1.6$ ppm from initially $2.7 \times 10^6 \pm 0.2$ ppm) (**Fig. 14a**). However, there was no increase in DOC concentration (**Fig. 14b**). The concentration of CH_4 was below the detection limit. In the FA-Fixed + NaN_3 setup, there was an increase in the CO_2 concentration while the CH_4 was also below the limit of detection. Likewise in the other setup, the DOC decreases until day 50 and then went back to the initial

value. The positive controls, those with the respective carbon source and without BIOS, the DOC behaved similarly to the experiments, while the concentration of gases showed increased in CO₂ from 2.7 ± 0.2 ppm to 3.1 ± 0.2 ppm, and from 1.4 ± 2.5 to 2.4 ± 3.4 ppm in controls with FA-Fixed and Yeast, respectively (**Fig. 14c**).

Discussion

Enrichment of fermentative microorganisms

Effect of biomass treatment

We successfully enriched microorganisms from the phyla Armatimonadota, Bacillota, Bacteroidota and Halobacteria. The ever presence of *S. oneidensis* (7.6%) in the FA-Fixed enrichment illustrate the complexity of the biomass, compared to Auto, where there were negligible copies of *S. oneidensis*.

Armatimonadota was enriched only when using autoclaved and FA-Fixed cell biomass, but not in the Yeast treatment, stressing their capability to degrade complex carbon. Although only a few representatives of Armatimonadota are known, they are abundant (>1.95%) in deep-sea sediments and possess extracellular enzymes for complex carbon degradation (CAZymes, e.g. GH62, GH141, GH136) (Carlton et al., 2023). Bacillota was the most abundant phylum in all enrichments (63% in Yeast, 33% in Auto 55% in FA Fixed). This is not surprising because it comprises the class Clostridia (**Figs. 15, 16 and 17**), rich in fermentative microorganisms and widely used in industry due to these capabilities (Du et al., 2020; Nimbalkar et al., 2019; Ponsetto et al., 2024). The genus *Proteiniclasticum* (30.2%) (**Fig. 15**) is capable of protein degradation (Namirimu et al., 2022), while the genus *Clostridium* (8.32%) (**Fig. 17**) is used for biofuel production (Nimbalkar et al., 2019).

Within the phylum Bacteroidota, the most representative genus was *Acetobacteroides* (17.8 and 22.3 in FA-Fixed and Auto, respectively) (**Figs. 15 and 16**). This genus includes multiple aceto-genic bacteria and often occurs in biomass-degrading fermentative enrichments (Su et al., 2014). This can explain the increase in DOC concentration, by the production of VFAs. These VFAs allowed for methanogenesis to occur, explaining the increase in CH₄ concentration. In the enrichment with Yeast, the main genus of Bacteroidota was *Macellibacteroides* (8.1), which is also a known strict fermentative microorganism whose main products include acetate, propionate and succinate (Jabari et al., 2012).

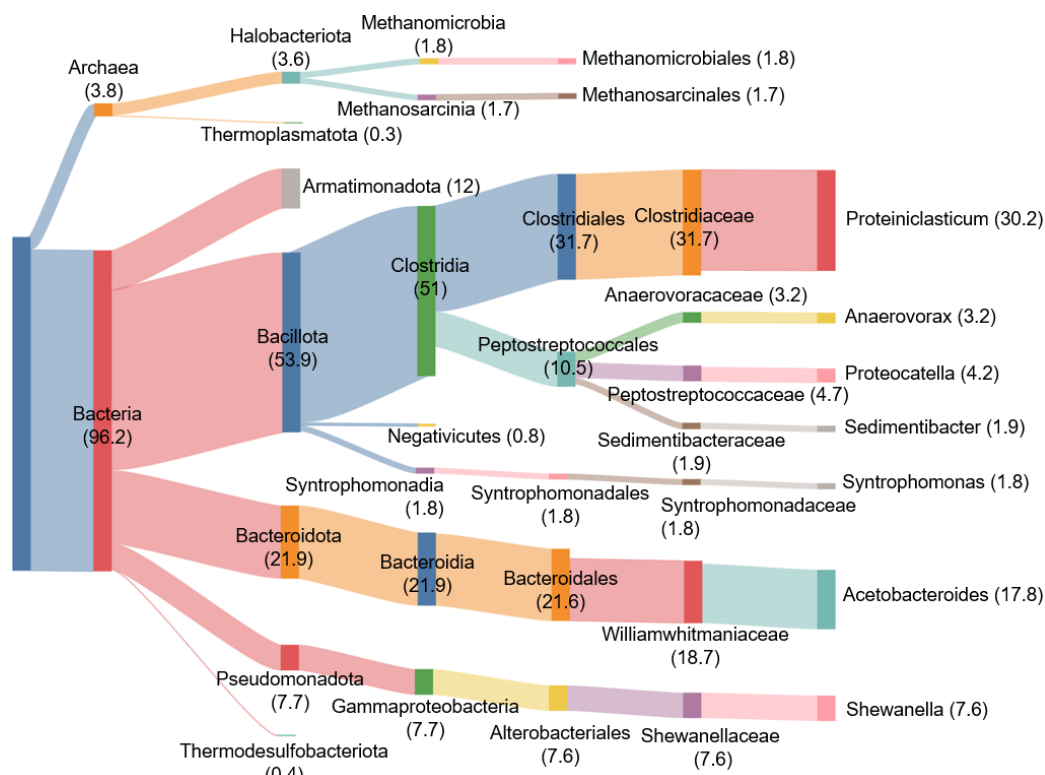


Figure 15. Sankey diagram illustrating the taxonomic abundances (% in parenthesis) of the identified microorganisms isolated using FA-Fixed biomass.

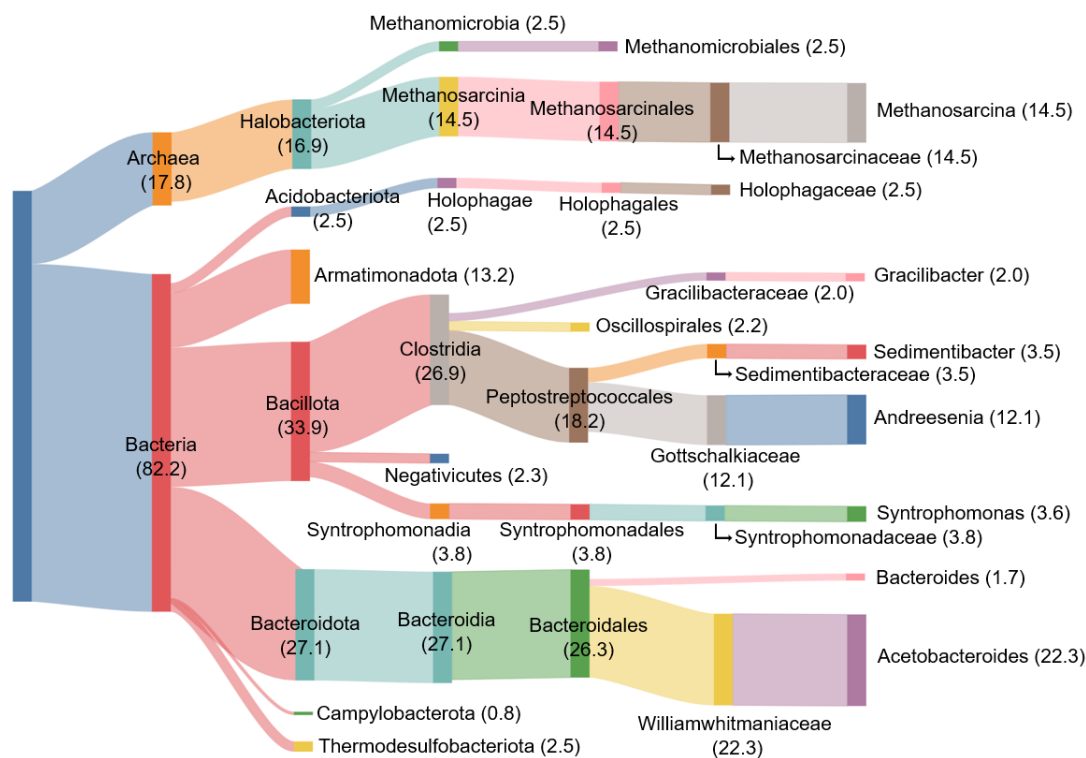


Figure 16. Sankey diagram illustrating the taxonomic abundances (% in parenthesis) of the identified microorganisms, isolated using autoclaved biomass.

Without additional carbon, significant changes can be seen only in the first transfer without NaN_3 , where the CO_2 concentration decreased and the CH_4 concentration increased. Because the first transfer contained the sediment, carbon could have been stored. However, with the transfers this carbon was lost. Therefore, in transfer 2 the production of CH_4 was one order of magnitude lower (~ 100 ppm) and then negligible in transfer 3.

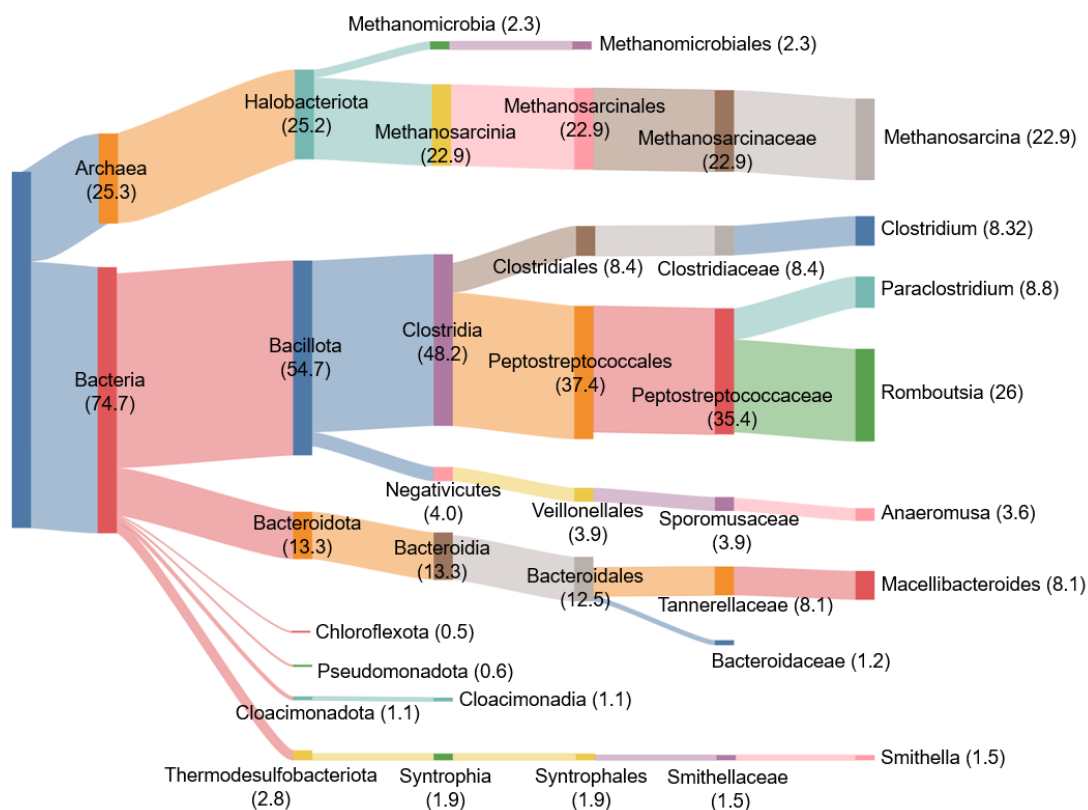


Figure 17. Sankey diagram illustrating the taxonomic abundances (%) of the identified microorganisms, isolated using yeast extract.

Effect of NaN_3

The setups with NaN_3 yielded mainly Pseudomonadota, which is associated with the *S. oneidensis* (dead) cells. The decrease in CO_2 concentration in transfer 1 of all treatments would suggest that there was biological activity, using CO_2 as carbon source. However, the decrease is not as pronounced in the other transfers, coupled with the increase in the relative abundance of Pseudomonadota (most likely *S. oneidensis*). Given the unchanged DOC concentration in Yeast + NaN_3 and considering that yeast is the most bioavailable carbon source among all setups, it is unclear whether the changes in the CO_2 and DOC concentration might have an abiotic origin. The degradation of *S. oneidensis* cells could explain the increase in the DOC concentration using Auto + NaN_3 and FA-Fixed + NaN_3 . This degradation, mainly organic acids, could cause a change in

the pH, leading to variation in the CO₂ concentration. This indicates that the inhibitor effect of NaN₃ blocked fermentation almost completely and not just anaerobic digestion.

Without additional carbon, only changes in the CO₂ concentration in transfers 1 and 3 were seen and could be related to CO₂ dissolution. It could potentially be biotic uptake of CO₂, but this did not lead to further C transformation as the DOC remained unchanged.

BIOS degradation

Fe(III) reduction

There was enrichment of phyla containing species of Fe(III) reducers, explaining the 21.8% reduction of the Fe(III) in the enrichment with FA-Fixed. Among the enriched microorganisms with FA-Fixed and Yeast (**Fig. 15 and 17**), order Methanosarcinales (1.7% and 22.9%) includes Fe(III)-reducers such as *Methanosarcina barkeri* MS (Bond and Lovley, 2002). With FA-Fixed there were also species of *Sedimentibacter* (1.9%) which can use the Fe(III) and could explain the difference in Fe(III) reduction, since they were absent in Yeast (Burkhardt et al., 2011; Chen et al., 2013). With Yeast, among the phyla with >1% of abundance, also Thermodesulfobacteriota (2.8%) contains representative genera of Fe(III) reducers (e.g., *Thermodesulfobacterium*, *Caldanaerobacter*) (Slobodkin et al., 1999). However, they are mainly thermophiles, and in the case of *Thermodesulfobacterium*, Fe(III) reduction is an alternative to the main metabolism. Then, there was no Fe(III) reduction in this setup. Finally, those enriched with NaN₃ did not show any reduction, expected by the inhibitory effect in respiratory electron transport (Lovley, 1993).

Use of BIOS -OM

Using the enrichments without NaN₃, there was CO₂ produced, although ~1x10⁶ ppm less than that produced in the controls and no CH₄ production. CO₂ may come from primary fermentative microorganisms (e.g., *Proteiniclasticum*, *Anaerovorax* or *Proteocatella*), whose fermentation produces CO₂, for instance by amino acid oxidation or decarboxylation reactions, confirmed by the increase of DOC. The lack of CH₄ using BIOS might be due to inhibition of methanogenesis by the Fe(III) (Bodegom et al., 2004). Although we cannot assign confidentially the CO₂ increase to degradation of BIOS-OM (due to its low concentration, 0.2%), we cannot discard its degradation, because of the increase in the CO₂ concentration (**Fig. 13a**). Future analysis, for instance with isotope-labeled carbon would help elucidating this unequivocally. In the controls, there was complete fermentation, including anaerobic digestion seen by the production of CH₄, which indicates that the enrichment worked and fermentative microorganisms are present.

The use of BIOS-OM by the enrichments with NaN_3 yielded a continuous decrease in DOC concentration during the first 50 days and then to an increase on the last day of incubation, suggesting the use of the available carbon first, for growth or storage followed by an accumulation of end products or cell degradation. In the case of Yeast, the fluctuation in CO_2 concentration could be caused by multiple processes, including consumption, production and carbonate chemistry. Acetogenesis and CO_2 incorporation could explain the initial and later consumption of CO_2 , while fermentation and oxidation of VFAs would produce CO_2 and explain the increase afterward.

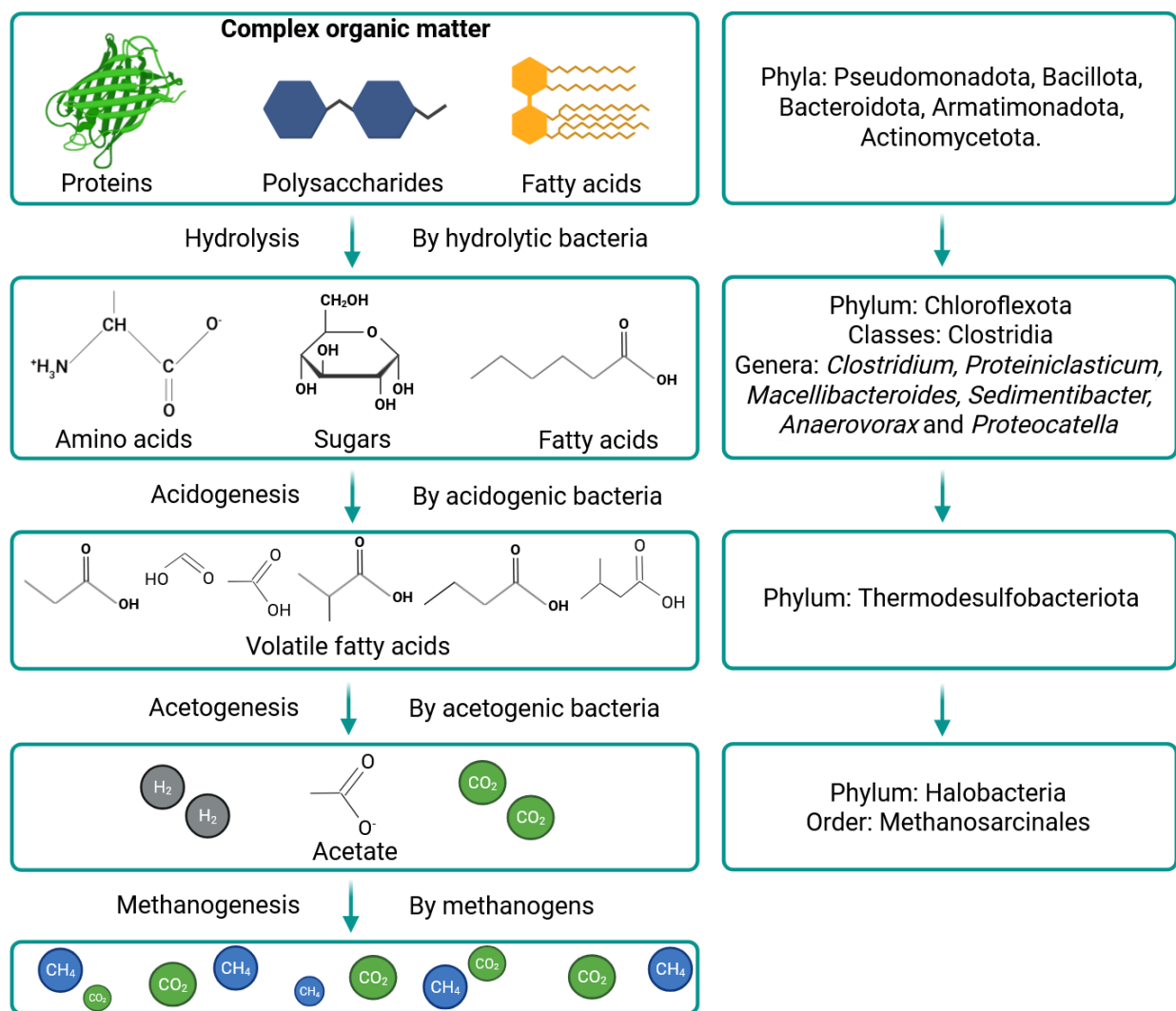


Figure 18. Overview of the fermentation process (left panels) and hypothetical fermentative network with the fermentative microorganisms enriched in this study (right panels).

Conclusions

Fermentative microorganisms were successfully enriched by using cell biomass from *S. oneidensis* after different treatments (autoclaved and FA-fixed) and yeast. Treatments without NaN_3 showed better performance, enriching the phyla Bacillota, Bacteroidota, Armatimonadota and Halobacteriota. These phyla include multiple genera with species capable of degrading complex organic carbon, including *Clostridium* and *Proteiniclasticum*, while Halobacteriota includes methanogens. This allowed complete fermentation (including anaerobic digestion) to occur, based on the appearance of methane in the headspace (**Fig. 18**). With NaN_3 the main phylum was Pseudomonadota, which we assumed comes from the cells of *S. oneidensis*, in the treatments with biomass, while it remained unchanged with Yeast.

When using the enrichments of FA-Fixed and Yeast with and without NaN_3 to degrade BIOS, only that with FA-Fixed showed reduction of Fe(III), not surprising due to the presence of genera of Fe(III)-reducers. Regarding the degradation of BIOS-OM, it cannot be completely ruled out as there were changes in the CO_2 concentration and DOC that cannot be completely attributed to abiotic processes. This indicates that despite the low-nutrient conditions, the enrichments showed microbial activity, encouraging follow-up work to constrain the use of BIOS-OM, for instance with isotopically labeled carbon.

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Chapter 6. Conclusions

Summary of results and discussion

BIOS are organo-mineral complexes produced by Fe(II) oxidizing bacteria when they oxidize Fe(II) to gain energy. They are composed of a mineral part and an organic part. The mineral part mainly consist of poorly crystalline Fe(III) (oxyhydr)oxides (e.g., ferrihydrite), while the organic part consists of polysaccharides and microbial cells. BIOS of different FeOB present different precipitation behavior of the minerals, strongly correlated to their metabolism. Nitrate-reducing FeOB typically exhibit high encrustation degree by the precipitation of minerals on the inner and outer cell membrane, while photoferrotrophs have developed strategies to avoid encrustation.

BIOS are environmentally relevant components of soils. They serve as electron acceptor for FeRB and can preserve organic carbon that could otherwise be transformed into greenhouse gases. Additionally, due to their unique morphologies and composition, some BIOS are considered promising biosignatures, in the form of microfossils or molecular indicators. In this thesis, I first provided an overview of the different mechanisms of Fe utilizing bacteria and their potential as biosignatures (Chapter 2). Then, I tested the bioavailability of BIOS_{Fe(III)} for FeRB (Chapter 3) and studied the minerals formed in detail in (Chapter 4) (**Figures 1 and 2**). Finally, I tested the bioavailability of BIOS_{OC} for different fermentative consortia that I isolated from sediment from Lake Constance (Chapter 5).

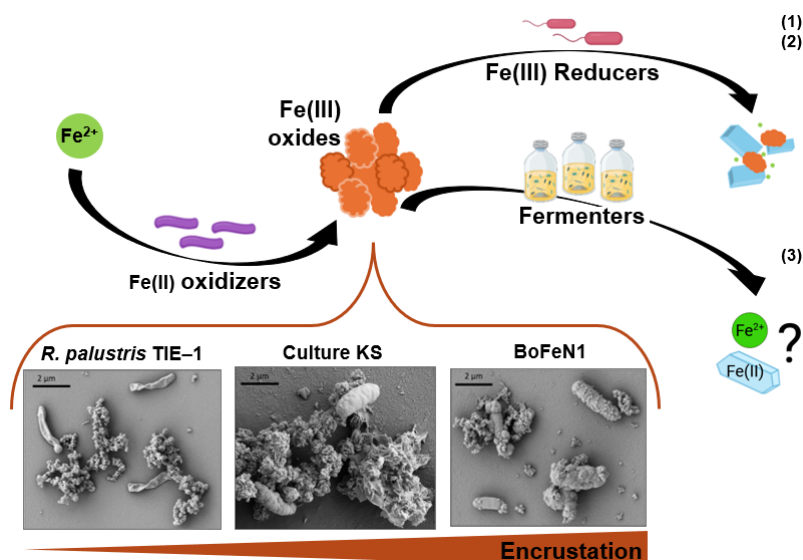


Figure 1. Overview of the objectives and results of the work presented here. The figure shows a sketch of how Fe(II) is oxidized by Fe(II) oxidizers that then produce Fe(III) oxides and depending on the strain, different degree of encrustation is observed. BIOS produced where exposed to degradation by Fe(III) reducers (1 and 2) and by fermenters (3).

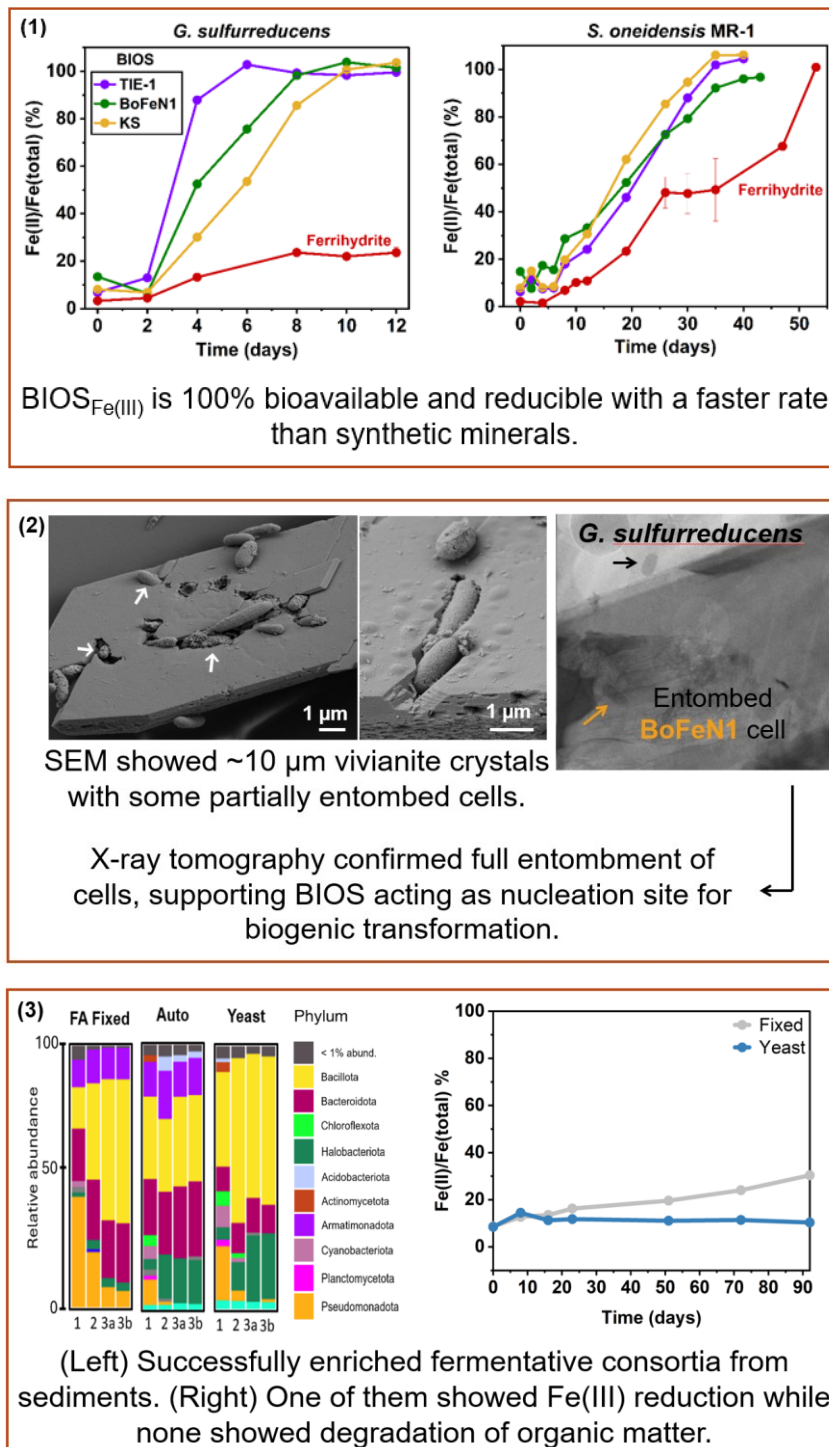


Figure 2. Summary of results. Numbers refer to those in figure 1, indicating the experiment.

BIOS as potential biosignatures

Fe is an essential element to living systems. It is ubiquitous on Earth and other planetary bodies within the solar system. I compiled a vast amount of literature in Chapter 1, summarizing what is

known about FeOB and FeRB, the different metabolisms for Fe(II) oxidation and Fe(III) reduction, the mineral products of each, and highlighting how they can be used as biosignatures and how these can help in the search of Fe-related biosignatures on Mars and icy moons.

BIOS of microaerophilic FeOB have been widely used as biosignatures due to their unique morphologies (Figure 3, Chapter 2). More importantly, these structures resist diagenetic processes with temperature and pressure up to 250°C and 140 MPa, respectively (Orange et al., 2014; Picard et al., 2015; Westall et al., 2015), granting a greater likelihood to be preserved in the geological record. In the future, the direction of research may investigate their preservation under extraterrestrial conditions to be able to compare to analogs or even extraterrestrial samples itself. This can be compared to Martian analog environments that are widely studied, including Iron Mountain (California), the Yilgarn Craton (Western Australia), Gunnuhver and Hveravellir (Iceland), Río Tinto, and the Atacama Desert (Azua-Bustos et al., 2022; Fernández-Remolar et al., 2021; Williams et al., 2021). Furthermore, with the current rovers on Martian soil and future sample return missions, the collection of experiments with lab-based BIOS and analogs will serve to compare to those samples and evaluate the potential biogenicity of some minerals or organics.

The exploration of those samples, however, poses challenges. Because the main differential element is morphological, their study requires techniques that are not always available on-board space missions, pushing the development or miniaturization of advance analytical tools and detections strategies. Ideally, high resolution techniques, for instance scanning electron microscopy, would be highly valuable to correlate cells and organic signatures with mineralogical structures.

Regarding icy moons, the evidence suggests ongoing hydrothermal processes in the interior of Enceladus and potentially Europa. Fe is a major component of hydrothermal systems on Earth and plays a key role in their biogeochemistry, in mineral formation and for biological activity. This opens multiple opportunities for the study of Fe-based biosignatures in icy moons with hydrothermal activity. Due to the inaccessibility and lack of in-situ measurements of these environments, it requires the development of robust proxies, analogs and adaptation to the current instruments. Main terrestrial analogs of icy moons typically include cold and redox-active environments, such as Lost City hydrothermal field, Blood Falls, lake Vostok and Svalbard lakes and glaciers. In terms of analysis, those included in current and future mission and that are relevant to studying Fe include Raman spectroscopy (Lopez-Reyes et al., 2014; Rull et al., 2017; Veneranda et al., 2020), infrared spectroscopy (Blaney et al., 2024) and mass spectrometry (Kempf et al., 2025).

Bioavailability of BIOS_{Fe(III)}

Using three representatives of FeOB (*Acidoborax* BoFeN1, culture KS and *Rhodopseudomonas palustris* TIE-1), I tested the bioavailability of their BIOS for two FeRB with different primary Fe(III) reduction mechanisms (direct contact by *G. sulfurreducens* and electron shuttles by *S. oneidensis*). Moreover, I tested BIOS after a freeze-thaw cycle, to simulate the effect of these conditions in high altitude environments.

Analysis of BIOS by Mössbauer spectroscopy revealed that the main components were ferrihydrite, goethite and lepidocrocite. In the case of KS BIOS there was also presence of vivianite, which is not biogenic but formed by abiotic precipitation of the initial Fe(II) and phosphate in the medium (Figure S3, Text S3, Table S1, Chapter 3). Micro-XRD analysis further confirmed the presence of goethite as the main crystalline mineral present (Figure S1, Chapter 3). Contrary to the similar mineral composition, the organic carbon present was significantly different TIE-1 > BoFeN1 > KS with 2.21%, 1.29% and 0.20%, respectively (Table 2, Chapter 3). Measurements of the particle size showed the particulates correlated with the presence of carbon. Then, the bigger particles in TIE-1 are attributed to aggregates formed using organic carbon as template, to prevent encrustation. the bigger size of BoFeN1 particulates compared to KS is attributed to the higher encrustation degree. Exposure to a freeze-thaw cycle to reflect environmentally relevant conditions to high altitude environments had a species-specific effect.

There were 24 setups in total, from 3 different FeOB, 2 different FeRB, 2 variables of presence/absence of additional organic carbon and 2 environmental conditions (fresh and freeze-thawed BIOS). To analyze how much the variation in the rate and extent of reduction depended on the setup variables, I performed a redundancy analysis. The results reveal that the presence of bioavailable organic carbon was the main factor controlling the reduction of BIOS_{Fe(III)} (Figure 5, chapter 3). When organic carbon was added to the medium in excess, all the BIOS_{Fe(III)} was reduced. Interestingly, this occurred at even higher rates than with lab-synthesized ferrihydrite, supporting the use of environmentally relevant minerals rather than synthetic analogs which could underestimate the reduction rates and capabilities in natural systems.

In alpine soils, glacier-associated sediments and other environments subjected to freeze-thawing, this process alters the physical properties of BIOS and their rate of reducibility. However, the mechanisms that govern these changes remain poorly understood. The changes in particle size

distribution, specific surface area (SSA), and total organic carbon (TOC) were FeOB-specific. Only the zeta potential consistently increased its magnitude towards more negative values in all BIOS, indicating an increase of organic moieties around the Fe nanoparticles. Altogether, the physical changes caused a decrease in the reduction rate, while the extent was only affected in reduction of TIE-1 BIOS used by *S. oneidensis*. Then, the presence of organic carbon was still the main factor controlling the reduction while freeze-thawing was a secondary factor. These results also indicate that the BIOS-associated organic carbon bioavailability is limited for FeRB. It might be too complex or inaccessible. Nevertheless, these organic molecules might still be bioavailable in natural systems, where other microbial groups could degrade them into simple organic molecules and/or greenhouse gases, such as fermentative microbes.

There was a significant difference in the reduction rate between the two FeRB, and the redundancy analysis confirmed that this was the main factor controlling the reduction rate. Two reasons could explain this difference. On the one hand, low molar OC/Fe ratios are known to favorize Fe(III) reduction by direct cell-mineral contact (Martinez and Alvarez 2018; Poggenburg et al. 2016). Consequently, with the molar OC/Fe ratios in the BIOS used in this work, ranging from 0.02 to 0.17, reduction by *G. sulfurreducens* was promoted. Moreover, this low carbon content, as compared to natural Fe–OC aggregates (<1.0 for sorption complexes and up to 6–10 for coprecipitation or chelation processes (Lalonde et al., 2012)), suggests that the role of BIOS in natural systems is more likely associated with absorbed or coprecipitated carbon, rather than native BIOS organic carbon. Yet, the role in organic carbon preservation might not be affected despite the origin. On the other hand, the reduction mechanism by *S. oneidensis* was disfavored by the lack of added electron shuttles. Compared to *G. sulfurreducens*, the lag phase of *S. oneidensis* is longer (Figures 3-4, Chapter 3), attributed to the time to either produce its own electron shuttles or to adapt to secondary reduction mechanisms (including direct cell-mineral contact).

Finally, the enhanced reactivity of BIOS compared to ferrihydrite is worth discussing. BIOS contain a mixture of ferrihydrite with crystalline minerals goethite and lepidocrocite. Because crystallinity is inversely proportional to reducibility at first glance BIOS would not seem more bioavailable. However, the results presented here show otherwise. The presence of organic carbon in BIOS is the main difference with synthetic minerals and might increase the redox reactivity of the minerals.

BIOS mineral transformation and implications in biosignature preservation

Biomineralization is a pathway to preserve biosignatures and in some cases a biosignature itself. Because some biominerals can resist long-term diagenetic processes (exposure to high

temperature and pressure) without being greatly altered (Li et al., 2013; Picard et al., 2015), they can preserve organic carbon embedded in the mineral structure. Moreover, in many cases they have unique morphologies that differ from their abiotically formed counterpart (Chan et al., 2009, 2009; Sievert et al., 2007). BIOS from microaerophilic FeOB are a good example of the unique morphologies that they can have (Figure 2, Chapter 2). However, they are exposed to microbial-mediated transformation.

Here, I exposed BIOS from three different FeOB (TIE-1, BoFeN1 and KS) to Fe(III) reduction by two species of FeRB (*G. sulfurreducens* and *S. oneidensis*) and studied the mineral transformation with microscopy techniques. In all cases, the reduction resulted in transformation of BIOS into vivianite (Figures S2 and S3, Chapter 4), leading to the complete loss of the initial morphologies. However, producing a new mineral envelope to protect and preserve the cells of FeOB. In the vivianite produced by both *S. oneidensis* and *G. sulfurreducens*, some cells were seen partially and fully entombed by the vivianite mineral. This was particularly marked after reduction of BoFeN1 BIOS, while less noticeable in those from KS and TIE-1.

This effect seems to be correlated to the initial degree of encrustation of the FeOB and suggest that the encrusted cells act as nucleation sites for Fe(III) reduction. Furthermore, more cells were seen entombed after BIOS_{Fe(III)} reduction by *G. sulfurreducens*, compared with *S. oneidensis*. This difference was attributed to the difference in the main reduction mechanisms. While direct mineral contact allows for Fe(II) to stay around the cells and most likely precipitate on the cells, for instance, attracted by the negatively charged cells, the use of electron shuttles typically occurs far from the cells, then reducing the likelihood of the minerals precipitating on the cells.

Due to the higher amount of cell entombment after reduction of BoFeN1 BIOS, further analyses were carried out, including FIB and X-ray tomography coupled to fluorescence microscopy. FIB revealed cells completely entombed in vivianite minerals and was confirmed by X-ray tomography. Meanwhile, the correlation of X-ray tomography with fluorescence microscopy suggests that the embedded cells might be FeOB, although this requires further systematic analysis to confirm which cells are embedded.

These findings have important implications. It suggests that despite microbial transformation of the minerals and the loss of the initial mineral biosignature, a portion of the cells remained entirely preserved within the minerals. Additionally, this place vivianite as an additional mineral phase

where microbial biosignatures can be found, relevant to lacustrine and other Fe-rich environments, including the recent report of possible presence of vivianite on Mars (Hurowitz et al., 2025). Moreover, it has implications for phosphate immobilization, relevant to early Earth studies as the availability of phosphorous limits microbial activity.

Isolation of fermentative microorganisms and test of BIOS_{oc} bioavailability

Isolation of microorganisms is an important task in microbiology. Despite the advanced omics approaches, isolation remains important to determine physiology and ecological function of different microbial species, because omics have limitations to confirm active pathways depending on the environmental conditions and interactions. Here, I successfully enriched fermentative microorganisms using *S. oneidensis* biomass subjected to different treatments (autoclaved and formaldehyde-fixed) and yeast, as carbon source. The most abundant phyla were Bacillota, Bacteroidota, Armatimonadota, and Halobacteriota, among which there are known taxa that are capable of degrading complex organic substrates. *Clostridium* and *Proteiniclasticum*, for instance, are widely used in the food industry, and Halobacteriota includes methanogenic microorganisms, widely used in the production of biofuel. The presence of methane in the headspace confirmed that complete fermentation, including anaerobic digestion. Worth mentioning, species from Armatimonadota are less known, then the enrichments from this work might offer the possibility to isolate and study new species as well interactions.

All the successful enrichments were cultured in the presence of BIOS from KS as sole carbon source to test the bioavailability of BIOS-associated organic matter. Although variations in CO₂ concentration and DOC were observed and overall suggest microbial activity, the breakdown of these molecules by abiotic processes could not be conclusively excluded. Interestingly, Fe(II) oxidation was observed in the setup with formaldehyde-fixed biomass, consistent with the presence of Fe(III)-reducing taxa within these communities.

General conclusion

Previous studies have found that the Fe(III) in naturally occurring BIOS from microaerophilic FeOB (e.g., stalks and sheaths) is bioavailable for microbial reduction (Langley, et al., 2009; Najem et al., 2016). In this thesis I systematically studied the bioavailability of both the Fe(III) and the C associated to BIOS formed by FeOB with different metabolisms (photoferrotrophy, chemototrophy and mixotrophy) under normal conditions of formation and of BIOS that undergo

freeze-thawing, to simulate other relevant environmental conditions, such as those that occur in permafrost.

The mineral part of the BIOS (>90% by weight) of the three FeOB used here (TIE-1, BoFeN1 and culture KS) was mainly composed of poorly crystalline Fe(III) oxides, goethite and lepidocrocite. The Fe(III) was available to 100% extent in the presence of additional organic carbon. In the lack of additional carbon sources, the BIOS_{Fe(III)} was bioavailable to a lesser extent (<52%). Moreover, the reduction rate was slower with BIOS subjected to freezing beforehand (**chapter 3**).

Interestingly, the reduction of BIOS_{Fe(III)} led to the entombment of the FeOB cells by the newly formed Fe(II) minerals (vivianite) and was significantly faster by *G. sulfurreducens* (direct cell-mineral contact reduction) than with *S. oneidensis* (electron shuttles). I confirmed the entombment of the cells using a combination of high-resolution fluorescence microscopy and soft X-ray tomography under cryogenic conditions (cryo-SIM-SXT). The results suggest that the Fe(III) (oxyhydr)oxides in the cell wall of FeOB act as nucleation sites for the formation of Fe(II) minerals that entomb and preserve the cells. This is of great interest for the preservation of organic matter and research in biosignatures (**chapter 4**).

Finally, I found that the BIOS_{OC}, which is in general cells and extracellular polymeric substances, was not completely bioavailable for FeRB. Therefore, I enriched fermentative microorganisms to test the degradation of BIOS_{OC}. I successfully enriched fermentative microorganisms from sediments from Lake Constance using different carbon sources (yeast extract, autoclaved biomass and formaldehyde-fixed biomass). The enrichments contained members of the phyla Bacillota, Bacteroidota, Armatimonadota and Halobacteria, which include known fermentative bacteria. However, the utilization of BIOS_{OC} as carbon source could not be confidentially demonstrated, and more tests will be needed to establish its bioavailability (**chapter 5**).

In general, the results of this thesis expanded our knowledge of the reactivity of BIOS. They added to our understanding of the role of microbes in the Fe cycle and its link with the C and P cycle. It also provided new pathways for organic matter preservation in the rock record. Overall, significantly contributing to the fields of geochemistry and geomicrobiology. However, multiple research gaps remain open and new perspectives are also proposed based on these results.

Outlook

Although this work demonstrates the bioavailability of the BIOS_{Fe(III)} from three strains with different Fe(II) oxidation metabolism, questions regarding the bioavailability of the associated carbon remain open. Although it was not bioavailable for the strains of FeRB used here, it could be bioavailable for other strains of FeRB or fermentative microbes. Furthermore, the enrichments of fermenters that resulted from this work open multiple opportunities for new the isolation of new species (Figures 16-18, Chapter 5).

Fe(III) (oxyhydr)oxides are known for their absorption capabilities. They immobilize trace elements and bind organic matter which helps to store carbon in critical environments, such as marine sediments. The findings of this thesis highlight the role of biogenic (oxyhydr)oxides in the intersection between the Fe and C cycle, with relevance in environmental, geological and astrobiological studies. On one hand, they serve as electron acceptors for FeRB. On the other hand, they help in the preservation of organic matter by encrustation or by entombment of cells. However, several questions remain open and are opportunities for future research.

First, an important knowledge gap worth studying are the mechanisms controlling the degradation of BIOS-associated organic carbon. The incomplete reduction of BIOS_{Fe(III)} in the absence of additional external carbon sources strongly suggest that this organic carbon is structurally complex or somehow protected for microbial degradation. Future studies in this direction may focus on identifying the precise molecular composition of the BIOS-associated organic carbon with high resolution instruments and techniques. Moreover, determine the microbial communities and the environmental conditions that promote their degradation. A combination of isotopic labeling approaches with metagenomics could provide insights into the pathways involved in the turnover of BIOS-associated organic carbon.

Another area for further investigation is the role of BIOS mineralogy and microbial origin in controlling BIOS bioavailability. Although this work reports an extensive investigation on this matter and demonstrates differences in reduction rates depending on the FeOB and FeRB, the precise relationship between these variables is not yet completely understood. Using additional representatives of both FeOB and FeRB, as well as other environmentally relevant microbial communities, will improve our understanding of the bioavailability of BIOS in relevant systems where they occur (e.g., soils, sediments, hydrothermal environments).

Finally, the observation of FeOB cells by vivianite precipitation that entombs the cells opened a new perspective in terms of fossil precipitation and (formation of) mineral biosignatures. Future work should test the stability of these mineralized cells in the long-term, simulating diagenetic conditions. This will determine the long-term stability and better assess their preservation potential in the geological record. This will have implications for a better interpretation of the geological record, as well as providing new biosignatures that can be looked for in Fe-rich environments beyond Earth, such as Mars. Additionally, the stability of the biogenic vivianite would have implications in the mobility of phosphate, impacting the biogeochemical cycle of phosphorous. Therefore, further studies of the transformation of BIOS and their role in the mobility of phosphorous can have significant impact in environmental remediation.

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Statement of personal contribution

The thesis was carried out in the frame of the Deutsche Forschungsgemeinschaft (DFG) project *Formation of Biogenic Iron-Oxides (BIOS) and their susceptibility to microbial iron reduction and fermentation under anoxic conditions*. The project number 494840258 was awarded to Dr. Muammar Mansor (applicant) and Prof. Dr. Andreas Kappler (co-applicant). Dr. Muammar Mansor wrote and designed the project with Prof. Dr. Andreas Kappler. The experiments were conceptualized by the three of us. I carried out experiments and wrote the manuscripts. Dr. Muammar Mansor helped with the writing, and Dr. Muammar Mansor and Prof. Dr. Andreas Kappler revised the final versions.

Chapter 2. I conceptualized the review. M. Mansor and A. Kappler acquired the funding. I wrote the original draft of the manuscript and all authors contributed to the review and editing of the final manuscript.

Chapter 3. M. Mansor and A. Kappler conceptualized the project. M. Mansor and A. Kappler acquired the funding. M. Mansor, A. Kappler and I designed experiments. I performed the experiments. Tsz (Alex) Ho Chiu carried out the XRD and BET measurements. Nora Kainz, Eva Voggenteiter and I measured DOC concentration. Marie Mollenkopf and I ran the HPLC. TOC measurements were done by Bernice Nisch. Dr. Jeremiah Schuster and I acquired the SEM micrographs and interpreted it. I analyzed the data with help of M. Mansor and A. Kappler. I wrote the original draft of the manuscript and all authors contributed to the review and editing of the final manuscript.

Chapter 4. M. Mansor conceptualized the project. M. Mansor and A. Kappler acquired the funding. Design of experiments by M. Mansor, C. Escudero and myself. I performed the growth experiments, C. Escudero performed the hybridizations and M. Wilsch-Bräuninger obtained the SEM images. M. Mansor, C. Escudero and I obtained the cryo-SIM SXT data. M. Mansor, C. Escudero, M. Wilsch-Bräuninger and I analyzed the data. I wrote the original draft of the manuscript and all authors contributed to the review and editing of the final manuscript.

Chapter 5. M. Mansor and A. Kappler conceptualized the project. M. Mansor and A. Kappler acquired the funding. Design of experiments by M. Mansor, A. Kappler and myself. Jennifer Lorenz obtained the enrichments under the supervision of M. Mansor and I. Jennifer Lorenz, Nora Kainz and I measured DOC. Jennifer Lorenz, Marie Mollenkopf and I ran the HPLC. I analyzed

the data with Jennifer Lorenz, M. Mansor and A. Kappler. I wrote the original draft of the manuscript and all authors contributed to the review and editing of the final manuscript.

I hereby state that I have not plagiarized or copied any of the text. Chapter 2 has been published in Earth-Science reviews and Chapter 3 has been published in Geo-Bio interfaces. Chapter 4 is still in the publication process, and the final published version of the manuscript may moderately differ from the version in this thesis.

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